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(54) Title: METHODS TO IDENTIFY MODULATORS OF CELL SIGNALING

(57) Abstract

Methods for assessing libraries of candidate modulators of intracellular signaling transmission pathways are described. The methods assess the ability of candidates from the library to inhibit the binding of peptides which represent participants in the signaling pathways as either a signal-generating protein or its cognate partner binding at a noncatalytic site. Specific peptides useful in this regard have been identified. In one application, modulators of the immune system can be identified by determining the ability of candidate substances to affect the interaction of PKC-theta or a fragment thereof with its cognate. The interaction can be measured using binding of the cognate as an index, or can be measured using a physiological response.

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METHODS TO IDENTIFY MODULATORS OF CELL SIGNALING

Technical Field

The invention relates to intracellular signal transduction. More specifically, it concerns methods to identify modulators of intracellular signal transduction by assessing the ability of candidate modulators to affect the interaction between a catalytically active signal-generating protein and a cognate binding protein involved in modulating the signal transduction function. The invention also concerns specific embodiments of such methods and the components thereof. In one illustration the methods are applied to identification of pharmaceutical agents for use in modulating activities of the immune system and methods of using agents identified in the disclosed methods.

Background Art

PCT Application WO 95/21252, discloses and claims peptide compositions which alter the activity of a signal-generating protein with respect to its cognate protein wherein the cognate protein contains at least one WD-40 region which putatively interacts with the signal-generating protein. The peptide compositions mimic the WD-40 regions, thus competing with the interaction of the cognate with the signal-generating protein. This competition results either in inhibiting the signal-generation or activating it.

One specifically exemplified signal-generating protein is protein kinase C (PKC); the illustrated cognate receptor for activated kinase C (RACK), in this case specific for βPKC, was designated RACK1. The gene encoding RACK1 was cloned and sequenced, showing that RACK1 contains the requisite WD-40 regions.

The above PCT application further describes methods to identify additional pairs of signal-generating proteins and their cognates and methods for recognizing WD-40 sequences in the cognates. This application also notes that such interactions can be used as a system to identify additional molecules that bind the signal-generating

protein by measuring the effect of candidate binding molecules on the interaction between the signal-generating protein and either its cognate *per se* or the polypeptide compositions that mimic the WD-40 regions of the cognate

Here, several specific peptides are identified that bind either to the signal-generating protein or to the cognate protein in a signal-affecting manner. The use of the signal-generating protein/cognate system to assay for modulators of signal transduction in assays which are independent of the purity of these participants is also described The PKC enzyme system is illustrated as a specific embodiment. In addition, peptides which reside on the signal-generating protein, as well as those which reside on the cognate or mimics thereof, are described as being useful to modulate the signal-generating interactions and biological activities which are mediated by the signal-generating interactions.

Further experiments that demonstrate the identity of a cognate protein for PKC-theta as the *fyn* protein are described. Since it is well established that *fyn* is involved in mediation of T-cell responses, it is apparent that disruption of the interaction of PKC-theta with its *fyn* cognate mediates the immune response, and that PKC-theta is a mediator of the immune response. Substances which can be shown to disrupt the interaction between PKC-theta and its cognate *fyn*, or, as described hereinbelow, to influence the interaction of PKC-theta with any cognate also have immunomodulating activity. The identification of *fyn* as a PKC-theta cognate, and the consequences of interfering with this association, demonstrate that PKC-theta is a significant signaling protein involved in the immune response.

Disclosure of the Invention

The present invention is directed to an efficient assay system to identify modulators of intracellular signaling pathways. Because the method takes advantage of inherent biological specificity, it can be conducted on impure preparations of the participants in the signal pathway -- the signal-generating protein and its cognate receptor controlling the signal pathway. The assay is conducted by assessing the interaction between the signal-generating protein and its cognate either by measuring

binding directly or by measuring a physiological or metabolic effect. The measurement is made in the presence and in the absence of a candidate modulator. Successful candidates which agonize the signal effect an increase in a metabolic or physiological output; antagonists effect a decrease. Both antagonists and agonists compete for binding between cognate and signal-generating protein.

Among successful candidates will be peptides which mimic regions on either the signal-generating protein or the cognate as well as nonpeptide small molecules. Due to their ease of identification, these peptides are particularly useful in alternate forms of the screening assays that detect binding between the peptide and the signal-generating or cognate protein. Although the assay methods disclosed may not all be suitable for direct screening of large chemical libraries, they do enable a sophisticated screening of candidates that can be combined with other techniques for selecting leads.

The methods described herein may involve peptides derived from the cognate or signal-generating protein. By "derived from" we mean that such peptides are either found in the cognate or signal-generating protein, or are modified by a limited number of conservative changes. Preferably the conservative changes represent less than about 20%, more preferably less than about 10%, and most preferably less than about 5% of the residues in the identified native sequence. One way to identify a suitable peptide is to compare sequences among species as is described in Example 4.

The invention is also directed to methods to screen libraries of candidate modulators using the above-described methods and to peptides representative of sites on the signal-generating protein and cognate which are themselves useful in these assays as well as in other applications involving the relevant interaction.

The present invention is also directed to specific embodiments in which the above disclosed methods are employed using PKC-theta protein, and its fragments or derived peptides along with the cognates of PKC-theta. The *fyn* protein and certain fragments thereof were found to bind to PKC-theta. Utilizing this interaction and that of PKC-theta and its fragments with other cognates, the present invention provides methods to identify immunomodulators by detecting modulators of biological and pathological processes which are mediated by PKC-theta/cognate interaction. Such

substances are particularly useful in modulating an activity of the immune system, particularly the activity and differentiation of T-cells.

Thus, at least one aspect of the invention is based on using the interaction of PKC-theta with its cognates as an index to substances that modulate activities of the immune system. In the Examples, evidence is presented that the *fyn* protein binds to PKC-theta. This observation is important because no binding partners of PKC-theta were known prior to applicants' invention. By contrast, *fyn* had previously been found to be associated with several T-cell receptor affiliated proteins, including CD3 zeta chain, ZAP-70 and Grb-2. The identified *fyn*/PKC-theta interaction of the present invention can be used as a basis for making and identifying agents which can modulate immune responses. Competitive assays using PKC-theta and the *fyn* peptide, or a PKC-theta or *fyn* equivalent, can be used to identify compounds which block *fyn*/PKC-theta interaction. A PKC-theta or *fyn* "equivalent" represents a peptide that can mimic the interactive binding activity of PKC-theta or *fyn* which is derived from the amino acid sequences of the appropriate binding regions. In addition, surrogate cognates can be used which may have no structural relationship to the *fyn* protein, but which bind PKC-theta in a manner similar to that displayed by *fyn*. Additionally, peptide and protein modeling techniques can be used to study the specific interactions of the cognate partners with PKC-theta to rationally design or rationally select agents for testing. Successful agents can be used as therapeutics to inhibit or otherwise modulate immune responses.

Brief Description of the Drawings

Figure 1 shows, diagrammatically, the known general primary sequence and domains of various PKC isoenzyme families.

Figure 2 shows the result of experiments demonstrating that PKC mediated effects on contraction of cardiac myocytes is inhibited by a fragment of the regulatory domain of εPKC but not by a corresponding fragment of δPKC.

Figure 3 shows the specific effect of an 8 amino acid peptide derived from the RACK-binding site in the regulatory domain of εPKC on the contraction rate of cardiac myocytes; an analogous peptide from the βPKC has no effect.

Figure 4 shows the specific effect of peptides derived from the RACK-binding site of βPKC on maturation of *Xenopus* oocytes.

Figure 5 shows the effects of peptides derived from RACK1 on PKC mediated maturation of *Xenopus* oocytes.

Figure 6 shows the effect of various peptides on the binding of θPKC V1 fragment to RACK1 *in vitro*.

Figure 7 is a photocopy of a blot obtained from a gel electrophoresis performed on proteins precipitated with antibodies raised against the V1/C1 boundary region of PKC-theta and probed with antibodies to phosphotyrosine and to *fyn*.

Figure 8 shows the regions of *fyn* used in a yeast two hybrid system and the resulting interactions. In Figure 8, ++ is a strong interaction (color development in <2 hr), + is a weak interaction (color development within 12 hours), and — is a null interaction (no color at 24 hours). U refers to the Unique sequence portion of *fyn*; SH3 and SH2 are the *src* homology domains; kinase is the conserved tyrosine protein kinase catalytic domain.

Figure 9 shows a detailed map of sequence motifs present in *fyn*, including the WD40 repeats, smaller RACK1 homologies, and the most conserved consensus PKC phosphorylation site.

Figures 10A and 10B show that the T2 peptide (aa 36-46) from the V1 region of PKC-theta reduces translocation of PKC-theta, but not of PKC-beta, from the soluble to the particulate fraction after activation of Jurkat T-cells with PMA/PHA (Figure 10A); peptide is taken up spontaneously from the medium in Jurkat T-cells. In parallel experiments, T2 peptide reduces expression of IL-2 as measured by ELISA in supernatant from stimulated cells (Figure 10B).

Figures 11A and 11B are photocopies of a blot which shows the effect of activation by various agents on translocation of PKC -theta and PKC-beta.

Figure 12 is a photocopy of a blot which shows the effect of TER14687 on PKC-theta translocation.

Figure 13 shows the results of binding of peptides encoded by the *fyn* kinase domains and two surrogates, clones 2-10 and 2-32, to PKC-theta V1 in the absence or presence of TER14687 using a two-hybrid system assay.

Figure 14 shows the effect of TER14687 on anti-CD3-induced production of IL4, IL5 and γIFN.

Figure 15 shows the nucleotide sequences and encoded amino acid sequences for clones 2-10, 2-32, #10, 1-22, 2-18, 2-20 and 3-1.

Modes of Carrying out the Invention

General Background

One aspect of the invention is, perhaps, best understood as a generalization of an illustrative interacting signal-generating pair, wherein the signal-generating protein is PKC and the cognate is an appropriate RACK. A peptide that mimics a PKC binding element contained in the RACK or in the PKC or that mimics a RACK-binding element contained in the PKC can be used as a component of assays relevant to the signal pathway.

PKCs represent a family of signal-generating isoenzymes, at least several of which are present in most cell types. Upon activation by a suitable agent, typically phosphatidylserine (PS) and diacylglycerol (DAG), and in some cases calcium ion, a PKC is translocated subcellularly, generally from the soluble fraction to another location in the cell that is associated with the particulate fraction. Each isoenzyme in this family apparently has one or more cognates (or RACKs) which are anchoring proteins at the appropriate locations associated with the physiological or metabolic effect of the activation of each particular isoenzyme. Thus, for example, one or a subset of PKCs contained in cardiac myocytes, when activated, results in a slowing of the contraction rate. One or a subset of PKCs contained in *Xenopus* oocytes, when activated, effect maturation of the egg. One or a subset of PKCs, when inhibited at the

catalytic site, blocks T-lymphocyte activation. See, Birdchall *et al. J Pharm. Expt'l Ther* (1994) **268**:922. The interaction of a particular PKC isoenzyme with its cognate RACK is required for the metabolic or physiological effect; therefore interference with this interaction will modulate that effect. Alternatively, the effect of the modulation may be agonistic if the interaction of the modulator promotes a conformational change in the signal generating partner corresponding to that normally occurring only upon the concurrent binding of activators (e.g., PS or DAG) and cognate protein, or otherwise results in signal activation.

The known PKC isoenzymes can be divided into three major groups as shown in Figure 1. All of the isoenzymes, regardless of group, contain a protein kinase domain represented by two constant (C) and two variable (V) regions. The regions which are responsible for the enzymatic activity are highly homologous or constant; the C4 region toward the carboxy terminus is thought to contain the catalytic site; the C3 regions upstream are responsible for binding ATP.

Upstream of the protein kinase domain in each case is a regulatory domain. All of the three families, the conventional (cPKC), the novel (nPKC) and atypical (aPKC) contain variable regions at the N-terminus designated V1, and constant regions immediately downstream marked C1. The C1 regions are thought to be involved in activation by phosphatidylserine, diacylglycerol, or pharmacological mimics such as phorbol esters. The C2 region is found only in the cPKC family and is thought to be the site for activation by calcium. However, the picture may not be quite so simple as C1 regions may also be involved in calcium binding, and the atypical class respond poorly to agents such as phorbol esters.

Nevertheless, it now appears clear that sequences within the regions shown as the regulatory domain are responsible for the interaction of the particular PKC with its cognate RACK. They may also contain a RACK-mimicking region, called a pseudo-RACK site, that prevents binding of PKC to its RACK when the PKC has not been activated. This situation is analogous to the pseudosubstrate sequence which is located elsewhere in the primary sequence and blocks the catalytic site prior to

activation of the PKC. It is shown hereinbelow that the relevant regions are specific for the particular isoenzyme involved in a designated signal-generation event.

In PCT application WO 95/21252, the cognate RACK1 protein which interacts with PKC-beta (a member of the cPKC family) was cloned and the WD-40 regions putatively responsible for binding to PKC-beta were identified through structural analogy. One of these WD-40 peptides was found to induce the kinase activity of PKC in the absence of PKC activators; both this peptide and another representing a WD-40 region rendered the PKC-beta susceptible to proteolysis, a characteristic of activated PKC forms. All of these peptides were also shown to inhibit the binding of PKC-beta to RACK1. In principle, the WD-40 regions of the appropriate RACK can serve as antagonists or agonists of the signal generation associated with the corresponding PKC. As described, an assay which shows the effects of members of a library of candidate modulators on interaction between the relevant PKC and its cognate or the relevant PKC and a WD-40 domain derived from said cognate can be used as a screening assay to identify modulators of this signal pathway.

Screening Assays

In the illustrative work described below, modulation of signal generation can be achieved by supplying, to a reaction mixture containing an appropriate PKC, either WD-40 peptides derived from the relevant RACK or pseudo-RACK peptides from the PKC regulatory domain which themselves mimic the RACK's binding domains, or a surrogate cognate and examining the effect of these peptides on binding or signal generation. Substances that interact -- i.e., behave as cognates -- will bind PKC and/or alter its signal generation. PKC or cognate-binding peptides derived from PKC can also be used as assay reagents in combination with the appropriate cognate to screen for modulators of the signal-generating pathway by virtue of the ability of the successful candidate to affect the binding of the cognate protein to the signal-generating protein. In general, systems which employ a PKC/cognate combination whose interaction can be observed can be used to screen for substances that affect this interaction. Thus, the interaction is observed in the presence of a candidate and in its

absence and the difference in the interaction under these two conditions reflects the ability of the candidate to modulate this interaction.

A particularly important signal-generating protein/cognate system is described hereinbelow. The signaling associated with PKC-theta is particularly significant in modulating the immune system, particularly T-cell responses. Therefore, substances that modulate the interaction between PKC-theta and its cognate are also modulators of the immune response, and thus can be used in contexts where modification of an immune response is desirable, such as in the treatment of allergies and asthma, preventing allograft rejection, and in autoimmune diseases. Described below are assay systems which employ PKC-theta or a fragment thereof wherein the cognate is that normally present in a host cell, is the *fyn* protein (the RACK in the above terminology) or a relevant fragment thereof, or a surrogate cognate defined according to the methods of the invention. As used herein, the terms "peptide" and "protein" will be used interchangeably without regard to size. It will be apparent in some cases which is intended according to conventional terminology; in others, both possibilities may be included. Applicants draw no arbitrary dividing line between peptides and proteins in terms of length of amino acid sequence.

Thus, in summary, various possible counterpart interactions can be tested; in no case are purified components required:

Component 1	Component 2
Signal-generating protein (e.g. PKC)	Endogenous cognate protein
"	WD-40 region of e.g. a RACK
"	Pseudo-RACK region of, e.g., PKC
"	Surrogate cognate
Cognate-binding region of, e.g., PKC	Cognate protein
"	WD-40 region of, e.g., RACK
"	Pseudo-RACK region of, e.g., PKC
"	Surrogate cognate

In the foregoing table, any assay method that detects the interaction appropriate to the components can be used. If the interaction is intracellular, certain

physiological effects on the cell can be measured. If the interaction is *in vitro*, direct measurement of binding of the cognate components to the signal-generating protein is probably most appropriate.

In general, the present invention is directed to screening methods to identify modulators of particular signal pathways. Each assay will involve a cognate that binds sufficiently and specifically to a catalytically active signal-generating protein, via a noncatalytic site, to permit assay in impure preparations. The interaction of these two components is observed in the presence and absence of a candidate modulator. Depending on the assay system chosen, the interaction and its modification can be observed in a variety of ways, including intracellular binding assays affecting an observable parameter; either a physiological readout, such as change in subcellular distribution, or an artificial construct, such as transcription of a reporter gene, can be used. In no case, however, are purified reagents required, although it may be convenient in some cases, for example, to utilize the peptides identified as illustrated below which represent regions of the signal-generating protein (illustrated by PKC-theta) or its cognate binding protein (represented by the *fyn*) that are responsible for interaction.

It will be noted that the relevant endogenous PKC and the relevant endogenous cognate can be used in observing the effects of candidate substances -- e.g., the ability of the candidate to alter intracellular translocation patterns upon activation of the signal-generating protein. Alternatively, surrogate cognate proteins can be used, such as those representing a WD40 region of a RACK or cognates identified using the approach described below by virtue of their ability to bind to the relevant PKC. If a surrogate is used in an *in vitro* system, generally binding is the measurable interaction. As used in the present application, the term "cognate" is applied to a substance that binds sufficiently and specifically to a catalytically active signal-generating protein via a noncatalytic site, as described above. "Cognate" therefore refers both to the endogenous cognate protein (or the relevant fragment) or equivalents derived from these and to a surrogate substance which shares the above-mentioned property. Although it is required that the cognate bind to a noncatalytic site, the cognate may, in addition, be a substrate for catalysis by the signal-generating protein.

As further described below, the peptides which can be substituted for one or the other component in the assay method are themselves identifiable through conduct of the assay. Thus, the ability of a compound to affect the interaction of the cognate protein and the signal-generating protein will identify it as a useful component of the assay, as well as a modulator of the signal pathway *per se*. Once the appropriate components are identified, the individual labeled components could be used to assess the level of binding. The labeled component may represent a region of the signal-generating peptide measured against a composition containing the cognate protein or, conversely, a component representing a portion of the cognate protein measured against the composition containing the signal-generating protein. These compositions may be whole cells or cell-free extracts or partially purified extracts.

It will be apparent that when a nonendogenous substance is chosen as one component of the assay, the screening tests are preferably performed by measuring only binding *per se*.

Alternatively, both the signal-generating protein and the cognate protein may be contained in a crude preparation and the method for assessing their interaction may include measuring localization of the signal-generating protein within the preparation *per se* or measuring a metabolic effect of the interaction, such as, for example, maturation of *Xenopus* oocytes or effect on the contraction rate of cardiac myocytes. The particular method of assessing the interaction will, of course, be appropriate to the partners in the interaction, and can readily be ascertained by taking advantage of the specificity of the signal pathways and their components as illustrated below.

In addition, the "two-hybrid" system may be used to effect interaction between a PKC or a fragment thereof and its cognate, and the effect of a candidate on this interaction can be observed. The "two-hybrid" system is described in U.S. Patent No. 5,283,173, incorporated herein by reference. Briefly, as applied to the present invention, a recombinant host, typically yeast, is transformed with two expression systems each encoding a fusion protein. One fusion protein contains a portion of a transcription-activating factor fused to a PKC or cognate-binding fragment thereof; the other fusion protein contains the complementary portion of the transcription-activating

portion fused to the cognate. Typically, the transcription-activating factor is an activator for RNA polymerase, and one portion represents the DNA-binding portion, the other the activator for the polymerase. When the cognate and PKC bind, the two portions of the transcription factor are brought into sufficient proximity that they are able to perform the function of activating transcription. The "two-hybrid" assay thus, also, will include a reporter expression system which is activated by the completed transcription factor to produce a reporter protein, such as β-galactosidase or chloramphenicol acetyl transferase. As defined herein, "two-hybrid assay" refers to this general approach.

For convenience, the assays to identify modulating candidate compounds will sometimes be described as measuring the effect of the candidate on the "binding" of the counterpart components in the reaction mixture. It will be understood that in the instance where both the cognate protein and the signal-generating protein are the active components of the composition participating in the assay, binding may be measured not only directly, but also by the resulting metabolic or physiological effects.

The *fyn*/PKC-theta Interaction

It has been shown hereinbelow that the signal generation mediated by PKC-theta is significant in regulating the activation of T-cells. Thus, modification of PKC-theta mediated signaling will result in modification of the immune response. The substances that can be identified as modulating this signaling pathway are thus useful as immunomodulating agents in preventing allergic reactions or reducing their severity, or in treating asthma, in ameliorating the effects of autoimmune disease, and in reducing the risk of rejection of transplants. It has also been demonstrated below that PKC-theta interacts endogenously with the *fyn* protein which serves as at least one of its endogenous cognates. Thus, the PKC-theta/*fyn* interaction *per se* can be used as an assay system for identifying candidate substances that will behave as immunomodulators.

In one form of the assay, the PKC-theta signaling protein and the *fyn* cognate occur endogenously in a test cell, and the effect of the candidate on the PKC signaling

function can be measured directly. As illustrated below, translocation of PKC-theta occurs on activation and thus binds to the endogenous cognate protein. The effect of a candidate substance on this translocation can be directly determined as a measure of its affect on interaction of PKC-theta with its cognate.

Alternatively, assays can be constructed *in vitro* or *in vivo* involving PKC-theta and *fyn* directly. Specifically, to identify an agent which modulates *fyn*/PKC-theta interaction, the *fyn* protein, a fragment of the *fyn* protein containing the domain which binds to PKC-theta or a fusion protein containing the domain of the *fyn* protein which binds to PKC-theta, is provided in the same environment with PKC-theta, a fragment of PKC-theta containing the *fyn* binding domain or a fusion protein containing the *fyn* binding domain, wherein the environment is such that ordinarily the signal-generating protein (PKC-theta) and the cognate (*fyn*) would interact. The environment is provided in the presence and in the absence of a substance to be tested. Differences in interaction between the signaling protein and the cognate in the presence and absence of the substance to be tested are compared; agents that block or otherwise affect the interaction can be identified by determining the differences in interaction between these two circumstances.

The *fyn* protein represents a family of splicing variants, and by "*fyn*" is meant any of these spliced variants and peptides which bind PKC-theta which are derived from them.

As used herein, an agent is said to block or decrease *fyn*/PKC-theta binding when the presence of the agent prevents or reduces the amount of association of the PKC-theta peptide with the *fyn* peptide. One class of agents will reduce or block the association by binding to the PKC-theta peptide while another class of agents will reduce or block the association by binding to the *fyn* peptide. Two examples of the first class of agent include antibodies which bind to the PKC-theta peptide and block the *fyn* binding site on PKC-theta and peptides which contain the PKC-theta binding site found on *fyn*. Two examples of the second class of agents include antibodies which bind to the *fyn* peptide and block the PKC-theta binding site on *fyn* and peptides

which contain the *fyn* binding site found on PKC-theta. Other types of interaction may also be envisioned.

The *fyn* peptide used in the present method can either be the entire *fyn* protein whose amino acid sequence is known in the art, a fragment of the *fyn* peptide which binds the PKC-theta, or a small region thereof that retains the binding activity or a protein which contains the PKC-theta binding site of *fyn*, such as a fusion protein containing the appropriate *fyn* sequence. Alternatively, the *fyn* peptide can contain more than one copies of the *fyn* sequence, such as in a palindromic or tandem repeat. A cell or virus expressing the *fyn* peptide can also be used.

As an alternative to compounds containing the *fyn* sequence, agents identified in the present method can be substituted for the *fyn* peptide. For example, an agent which is found to block *fyn*/PKC-theta binding by binding to PKC-theta can be used in place of the *fyn* peptide.

The PKC-theta peptide used in the present method can be any member of the PKC-theta family of proteins so long as the member binds the *fyn* peptide. As used herein, a PKC-theta family member refers to proteins currently known in the art which are members of the PKC-theta family of proteins (for a review see Baier *et al J Biol Chem* (1993) **268(7)**:4997-5004 and Baier *et al.*, *Eur J Biochem* (1995) **225(1)**:195-203). These include PKC-theta isolated from organisms such as humans, mice, etc., as well as the various splice forms of PKC-theta found in each organism. The PKC-theta family member can be used in its entirety or a fragment of the PKC-theta protein which contains the *fyn* binding site can be used. The preferred fragment will be derived from the V1 region of PKC-theta; the strongly isozyme-specific regions V3 or V5 also can be used. Alternatively, a cell or virus expressing the PKC-theta, or PKC-theta fragment, can be used.

The *fyn* and PKC-theta peptides used in the present invention can be used in a variety of forms. The peptides can be used in a highly purified form, free of naturally occurring contaminants. Alternatively, a crude preparation containing a mixture of cellular components as well as the *fyn* and PKC-theta peptides can be used. Further, the *fyn* or PKC-theta peptides can be isolated from cells which naturally express these

peptides, from cells which have been altered, using recombinant methods, to express these peptides, or can be synthesized using standard peptide synthesis methods. So long as the association of the PKC-theta peptide with the agent to be tested and/or the *fyn* peptide can be identified in the sample, the *fyn* and PKC-theta peptides are in a suitable form for use in the above described assay.

The *fyn* and/or PKC-theta peptides can additionally be modified to contain a detectable label or signal generation system to facilitate detection. Methods for attaching agents such as fluorescence tags or fluorescence polarization and secondary labeling agents such as biotin, are well known in the art.

A variety of art-known methods can be adapted and employed to detect whether an agent blocks or reduces the interaction of the *fyn* peptide with the PKC-theta peptide. Such methods include, but are not limited to, assays which employ a solid support, assays in solution phase, assays performed in a gel-type media, and assays which use a combination of these environments. An example of a solid phase assay would be one in which one or both of the *fyn* and PKC-theta peptides are immobilized on a solid support and is incubated in a solution phase with the agent to be tested and the other peptide of the *fyn*/PKC-theta pair. A secondary detection means, such as an antibody, is then used to determine the amount of the second peptide which binds to the immobilized peptide. Alternatively, the second peptide of the *fyn*/PKC-theta pair can be detectably labeled and its binding to the immobilized first peptide is directly assessed. One format which is preferably suitable for a solid phase based assay is immobilization of one of the peptides in a 96-well micro-titer plate. Such titer plates provide an efficient assay format for rapidly processing multiple samples.

Alternatively, both peptides of the *fyn*/PKC-theta binding pair can be in solution. After mixing, the binding of the *fyn* peptide to the PKC-theta peptide can be detected using a variety of methods, for example detecting mobility shifts using electrophoretic means. One skilled in the art can readily appreciate how numerous assay-type formats which are known in the art for use in competitive assays can be modified to use the *fyn*/PKC-theta peptide pair.

As described above, the binding of PKC-theta and *fyn* or a *fyn* substitute can be detected using the two-hybrid assay system. The "*fyn* substitute" can be any alternative peptide or other molecule which is found in the assays of the invention to bind to PKC-theta so as to interfere with the action of PKC-theta with *fyn* or other endogenous cognates. Thus, the discussion herein is not to be considered limited to PKC-theta/*fyn* interaction *per se*, but includes interactions of PKC-theta with any cognate.

Direct binding to the PKC-theta peptide or the *fyn* peptide can, but need not, be used as first step in identifying agents which block *fyn*/PKC-theta interaction. In such methods, agents are first screened for the ability to bind to the PKC-theta or *fyn* peptides. Agents which bind to either of the two peptides are then screened for the ability to block *fyn*/PKC-theta interaction, or for the ability to modulate a function of the immune system.

Agents which are assayed in the above methods can be randomly selected or rationally selected or designed. As used herein, an agent is said to be randomly selected when the agent is chosen randomly without considering the specific sequences involved in the association of the *fyn* peptide with the PKC-theta peptide. An example of randomly selected agents is the use of a chemical library or a peptide combinatorial library.

As used herein, an agent is said to be rationally selected or designed when the agent is chosen on a nonrandom basis which takes into account the sequence of the target site and/or its conformation in connection with the agent's action. As described above, two sites of actions for agents of the present invention are the *fyn* peptide and the PKC-theta peptide. Agents can be rationally selected or rationally designed by utilizing the peptide sequences which make up the contact sites of the *fyn*/PKC-theta pair. For example, a rationally selected peptide agent can be a peptide whose amino acid sequence is identical to the *fyn* contact site found on PKC-theta. Such an agent will reduce or block the association of *fyn* with PKC-theta by binding to *fyn*.

The agents of the present invention can be peptides, small molecules, vitamin derivatives, as well as carbohydrates. A skilled artisan can readily recognize that there

is no limit as to the structural nature of the agents of the present invention or those used in the present assay methods.

As provided above, one class of agents of the present invention is that of peptide agents whose amino acid sequences are chosen based on the amino acid sequence of *fyn* and in particular the PKC-theta contact site found on *fyn*, while a second class of agents is that of peptide agents whose amino acid sequences are chosen based on the amino acid sequence of PKC-theta and in particular the *fyn* contact site found on PKC-theta. The *fyn* contact site on PKC-theta and the PKC-theta contact site on *fyn* can readily be determined using art-known methodologies. For example, tryptic digestion of the PKC-theta protein can be performed and the various fragments of PKC-theta can be tested for their ability to bind the *fyn* peptide. Alternatively, a modification of a bind and chew assay can be used in which the *fyn* and PKC-theta peptides are allowed to interact and the interactive pair is subject to protein digestion. Regions of the PKC-theta peptide which are contacted by the *fyn* peptide will be protected from digestion and can be later characterized to determine the amino acid sequence which is bound and protected. Alternative assays which detect binding of the various fragments can also be used.

All of the peptide agents of the invention, when an amino acid forms the C-terminus, may be in the form of the pharmaceutically acceptable salts or esters. Salts may be, for example, Na^+, K^+, Ca^{+2}, Mg^{+2} and the like; the esters are generally those of alcohols of 1-6C. In all of the peptides of the invention, one or more amide linkages (-CO-NH-) may optionally be replaced with another linkage which is an isostere such as $-CH_2NH-$, $-CH_2S-$, $-CH_2CH_2$, -CH=CH- (cis and trans), $-COCH_2-$, $-CH(OH)CH_2-$ and $-CH_2SO-$. This replacement can be made by methods known in the art. Alternative peptide linking moieties can also be used to decrease the rate of degradation of peptide based agents. The following references describe preparation of peptide analogs which include these alternative-linking moieties: Spatola, A.F., Vega Data (March 1983), Vol. 1, Issue 3, "Peptide Backbone Modifications" (general review); Spatola, A.F., in "Chemistry and Biochemistry of Amino Acids Peptides and Proteins," B. Weinstein, eds., Marcel Dekker, New York, p. 267 (1983) (general review); Spatola, A.F., *et al.*, *Life Sci* (1986) **38**:1243-1249 ($-CH_2-S$); Hann, M.M., *J*

Chem Soc Perkin Trans I (1982) 307-314 (1982) (-CH-CH-, cis and trans), Almquist, R.G., *et al.*, *J Med Chem* (1980) **23**:1392-1398 (-$COCH_2$-); Jennings-White, C., *et al.*, *Tetrahedron Lett* (1982) **23**:2533 (-$COCH_2$-); Holladay, M.W., *et al.*, *Tetrahedron Lett* (1983) **24**:4401-4404 (-$C(OH)CH_2$-), and Hruby, V.J., *Life Sci* (1982) **31**:189-199 (-CH_2-S-).

In addition to analogs which contain isosteres in place of peptide linkages, the peptides or proteins of the invention include peptide mimetics in general, such as those described by Olson, G.L. *et al. J Med Chem* (1993) **36**:3039-3049 and retro-inverso type peptides as described by Chorev, M. *et al. Science* (1979) **204**:1210-1212; and Pallai, P.V. *et al.*, *Int J Pept Protein Res* (1983) **21**:84-92.

Another class of agents of the present invention is that of antibodies immunoreactive with critical positions of the *fyn* protein or with the PKC-theta protein. Since the target for action of the agents of the present invention is within a cell (cell signaling involved in *fyn*/PKC-theta interaction), antibody agents are most useful in immunodiagnostic methods and find use as substitutes for either the *fyn* or PKC-theta peptides in the present methods. However, using currently available recombinant technologies, antibodies can be produced intracellularly, and thus can participate in intracellular forms of the assays as well

Antibody agents are obtained by immunization of suitable mammalian subjects with peptides, containing as antigenic regions, those portions of the *fyn* or PKC-theta proteins which are intended to be targeted by the antibodies. Critical regions include, but are not limited to, the contact sites involved in the association of *fyn* with PKC-theta and sites which provide steric interference with the contact sites upon binding.

Antibody agents are prepared by immunizing suitable mammalian hosts in appropriate immunization protocols using the peptide haptens alone, if they are of sufficient length, or, if desired, or if required to enhance immunogenicity, conjugated to suitable carriers. Methods for preparing immunogenic conjugates with carriers such as BSA, KLH, or other carrier proteins are well known in the art. In some circumstances, direct conjugation using, for example, carbodiimide reagents may be effective; in other instances linking reagents such as those supplied by Pierce Chemical

Co., Rockford, IL, may be desirable to provide accessibility to the hapten. The hapten peptides can be extended at the amino or carboxy terminus with a cysteine residue or interspersed with cysteine residues, for example, to facilitate linking to carrier. Administration of the immunogens is conducted generally by injection over a suitable time period and with use of suitable adjuvants, as is generally understood in the art. During the immunization schedule, titers of antibodies are taken to determine adequacy of antibody formation.

While the polyclonal antisera produced in this way may be satisfactory for some applications, for pharmaceutical compositions, use of monoclonal preparations is preferred. Immortalized cell lines which secrete the desired monoclonal antibodies may be prepared using the standard method of Kohler and Milstein or modifications which effect immortalization of lymphocytes or spleen cells, as is generally known. (See Harlow: Antibodies Cold Spring Harbor Press NY 1989) The immortalized cell lines secreting the desired antibodies are screened by immunoassay in which the antigen is the peptide hapten or is the PKC-theta or *fyn* peptide. When the appropriate immortalized cell culture secreting the desired antibody is identified, the cells can be cultured either *in vitro* or by production in ascites fluid.

The desired monoclonal antibodies are then recovered from the culture supernatant or from the ascites supernatant. Fragments of the monoclonals or the polyclonal antisera which contain the immunologically significant portion can be used as antagonists, as well as the intact antibodies. Use of immunologically reactive fragments, such as the Fab, Fab', of $F(ab')_2$ fragments is often preferable, especially in a therapeutic context, as these fragments are generally less immunogenic than the whole immunoglobulin. The antibodies or fragments may also be produced, using current technology, by recombinant means. Regions that bind specifically to the desired regions of receptor can also be produced in the context of chimeras with multiple species origin.

It is, of course, unnecessary to conduct initial production of the antibodies through immunization and isolation of immortalized cells that secrete monoclonals. Techniques are available for cloning immunoglobulin genes so that production can be

effected through *de novo* recombinant synthesis. Regardless of whether the genes originate in immortalized B cells or result from cloning efforts, manipulation of the genes permits redesign of the immunoglobulins, including production of single-chain immunoglobulins such as F_V fragments.

Uses for Substances which Modulate Interaction of Cognate with PKC-theta

Interaction between PKC-theta and its cognate has been implicated in modulating a variety of biological responses. According to the present invention, this interaction results in modulation of the immune system, particularly those activities involving T-cell activity. Therefore, substances which affect PKC-theta interaction with its cognate can be used to modulate activities of the immune system.

Specifically, immune system activity, such as T-cell mediated responses, can be modulated by administering to a subject a substance which affects the interaction of an appropriate cognate with PKC-theta. The subject can be any vertebrate in need of modulation of immune activity. These substances are particularly useful in treating human subjects.

Immune system activity refers to the wide variety of cellular events in which cells of the immune system participate. Examples of situations where it is desirable to modulate such activity include, but are not limited to, transplant surgery, autoimmune disorders, and response to allergens. In each of these situations, it is desirable selectively to reduce T-cell responsiveness.

A substance modulates an immune system activity when it reduces the severity of a pathological condition mediated by the immune system, or when it affects either positively or negatively the normal immune activity of the subject. For example, an agent is said to modulate the immune system activity involved in graft rejection when it reduces the rate of onset of graft rejection or reduces the severity of graft rejection. Other effects on the immune system can be determined more precisely. For example, the resultant effect may be to diminish the production of cytokines such as IL-4 or IL-5 as illustrated below, preferably with no effect on γ-IFN production.

Administration of Agents which Modulate Immune System Activity

The agents of the present invention can be provided alone, in combination with another agent that modulates a function of the immune system, or in combination with drugs having additional physiological effects. For example, a substance of the present invention that reduces T-cell activity can be administered in combination with other immunosuppressive agents, and/or with another substance so identified. As used herein, two agents are said to be administered in combination when the two agents are administered simultaneously or are administered independently in a fashion such that the agents will act at the same time.

The agents of the present invention can be administered via parenteral, subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal, or buccal routes. Alternatively, or concurrently, administration may be by the oral route, or by inhalation. The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired.

The present invention further provides compositions containing one or more agents of the present invention. While individual needs vary, determination of optimal ranges of effective amounts of each component is within the skill of the art. Typical dosages are in the range of 0.01-50 mg/kg body weight. Preferred dosages are in the range of 0.1-10 mg/kg; most preferably 0.1-1 mg/kg.

In addition to the pharmacologically active agent, a composition comprising an agent of the present invention may contain suitable pharmaceutically acceptable carriers such as excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically for delivery to the site of action. Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form, for example, water-soluble salts. In addition, suspensions of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or

triglycerides. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol, and/or dextran. Optionally, the suspension may also contain stabilizers Liposomes can also be used to encapsulate the agent for delivery into the cell

The pharmaceutical formulation for systemic administration according to the invention may be formulated for enteral, parenteral or topical administration Indeed, all three types of formulation may be used simultaneously to achieve systemic administration of the active ingredient.

Suitable formulations for oral administration include hard or soft gelatin capsules, pills, tablets, including coated tablets, elixirs, suspensions, syrups or inhalations and controlled release forms thereof.

Suitable formulations for administration by inhalation include metered dose inhalers and dry powder devices. For nasal absorption aqueous and nonaqueous suspensions or dry powders may be used.

Methods for Targeting

The effective affinity of the substances of the present invention can be increased by covalently linking the agent to a second agent which also binds either PKC-theta or its cognate. Such a second agent will bind to another site on either the cognate or PKC-theta molecule and bring the first agent into close proximity to the target site, augmenting the overall avidity. Such second agents can be, but are not limited to, antibody and peptide agents. The second agent can be covalently attached to the first agent using art-known methods. Methods which employ linkers are particularly well suited for this use.

Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, practice the claimed invention The following working examples illustrate preferred embodiments of the present invention, and are not to be construed as limiting.

Example 1

Specificity of Negative Chronotropy for εPKC Translocation

Neonatal rat cardiac myocytes were used in this assay. These cells, when prepared in culture, exhibit contractions at approximately the rate of 40-50 /15 sec., and it is known that the phorbol ester, 4-β phorbol 12-myristate-13-acetate (PMA) reduces the contraction rate (Johnson, J.A. *et al.*, *Circ Res* (1995) **76**:654-653). Previous work has also shown that treating cardiac myocytes with PMA or with norepinephrine (NE) causes translocation of αPKC to the nuclear boundary, βIPKC to the interior of the nuclei, δPKC to the fibrillar and perinuclear structures, and εPKC to cross-striated structures (Disatnik, M-H. *et al.*, *Exp Cell Res* (1994) **210**:287-297). It has also been shown that exogenously added activated PKCs bind similarly (Mochly-Rosen, D. *et al.*, *Molec Biol Cell* (1990) **1**:693-706). Since the location to which the various isoenzymes are translocated are different, it has been suggested that the variable regions specific for each isoenzyme (Nishizuka, Y., *Nature* (1988) **334**:661-665) should contain at least part of the specific RACK binding site (Disatnik, M-H. *et al.*, *Exp Cell Res* (1994) **210**:287-297). Furthermore, it has been suggested that the V1 region of εPKC determines its substrate specificity (Pears, C. *et al.*, *Biochem J* (1991) **276**:257-260).

To show that only translocation of the corresponding isoenzyme is inhibited by one of its fragments -- e.g., only translocation of εPKC is inhibited by an εPKC-V1 fragment, cells cultured on chamber slides were permeabilized with saponin (50 μg/ml) in the absence or presence of 100 μg/ml rat recombinant εPKC-V1 or δPKC-V1 fragments containing amino acids 2-144 in each case. Cellular functions, including cell viability, spontaneous and stimulated contraction rates, gene expression and hypertrophy are unaffected by the saponin treatment.

These fragments were prepared by amplifying the relevant portion of the gene from a cDNA library (Stratagene). A FLAG™ epitope (DYKDDDK) was engineered at the 5' end of the fragment and the 0.45 kb PCR fragment was subcloned into pMAL-C2 vector (New England Biolabs) for overexpression as a fusion protein with maltose binding protein in *E. coli*. Protein purification and Factor Xa proteolysis of

the fusion proteins was as described by Ron, D. *et al.*, *Proc Natl Acad Sci USA* (1994) **91**:839-843

The intracellular concentration of each fragment was approximately 300 nM or about 3% of the extracellular concentration as determined by quantitative Western blot of washed and extracted cells

After the εPKC-V1 or δPKC-V1 fragments were administered by permeabilization, the cells were incubated with either 4-α or 4-β PMA. (4α PMA is not active and is used as a control.) The cells were then fixed with methanol and acetone and PKC isoenzyme localization was determined by immunofluorescence; the antisera used to detect δPKC and εPKC do not recognize the administered fragments. Multiple fields of cells for each treatment group and for PKC isoenzymes α, βI, δ, and ε were observed and the data were presented as a percentage of cells having the tested enzyme at the activated site. When the cells were treated with 100 nM PMA for five minutes, it was apparent that neither δPKC-V1 nor εPKC-V1 had any effect on translocation of the α or β isoenzymes whereas each of the δ and ε fragments specifically inhibited the translocation of the corresponding isoenzyme, but not the other isoenzyme. An additional experiment measuring translocation of εPKC at the much lower level of 3 nM PMA also showed complete inhibition by the ε fragment. It has previously been shown that 3 nM PMA is only marginally effective in translocation of PKC isoenzymes other than the ε form (Johnson, J.A. *et al.*, *Circ Res* (1995) **76**:654-663).

The localization of εPKC to the cross-striated structures suggested that the ε isoenzyme might mediate the effect of PMA on the contraction rate. Cells were cultured and permeabilized with saponin in the presence or absence of 150 μg/ml δ or εPKC-V1 fragments described above. Basal contraction rates were monitored for 10 min, and the cells were then treated with 3 nM PMA. The rate of contraction was monitored over the next 20 minutes. The results are shown in Figure 2

As shown, in cells where no fragment was added, the contraction rate is reduced almost to zero within 15 min of the addition of PMA. Similarly, in cells where the δPKC fragment is added, the contraction rate is thus reduced. However, in cells

where the εPKC fragment was added, the contraction rate is maintained. Thus, the εPKC-V1 fragments specifically prevented PMA-induced inhibition of spontaneous contraction. These data, combined with the data described above with respect to translocation and the fact that the εPKC-V1 fragment does not affect the catalytic activity of εPKC *in vitro*, demonstrate that the translocation of εPKC is an essential step in signaling the chronotropic effect of PMA and that this signaling is inhibited by a fragment containing the V1 region.

The effect of PMA in reducing the contraction rate can be mimicked by controlling the α1 and β1 adrenergic receptors of the myocytes, providing a more physiologically relevant phenomenon. If both the α1 and β1 receptors are activated with NE, an increase in contraction rate occurs; when both receptors are inhibited, NE no longer has this effect. If the α1 receptor is inhibited alone by prazosin, the initial increase in contraction rate is higher; if the β1 receptor alone is inhibited, the contraction rate decreases.

When either the δ or ε fragments described above is substituted for the known inhibitors of the α1 and β1 receptors, the behavior of the cells in response to NE is unaffected by the presence of the δ fragment; however, addition of the ε fragment gives a response similar to that obtained in the presence of prazosin. These data are consistent with the role of the ε fragment in controlling contraction rate since the α1 receptor (inhibited by prazosin) mediates PKC translocation.

Figure 3 shows the results of a similar experiment using stimulation with 3 nM PMA, and providing peptides of less than 10 residues that block localization of εPKC and βPKC using a 0.1 μM εPKC-derived peptide εV1-2 (sequence EAVSLKPT) or 20 μM of a βPKC-derived peptide βC2-4 (sequence SLNPEWNET). As shown in Figure 3, stimulation with 3 nM PMA without adding peptides to the permeabilized cells or in the presence of 20 μM of the βPKC localization inhibitor results in negative chronotropy as above.

Example 2

Specific Inhibition of βPKC Translocation by C2-Derived Peptides

The parent application herein described binding sites on a particular RACK, RACK1, which are responsible for binding βPKC. It is recognized that if the binding site on PKC is identified, peptides that mimic this binding site could also serve as modulators of βPKC translocation and function. Furthermore, it should be noted that PKC may itself contain pseudo-RACK peptide sequences that mimic the binding sites on RACK and regulate the exposure of the binding site for RACK on PKC. The following experiments do not distinguish between these possibilities; nevertheless, whichever function on the PKC sequence is represented, mimics of the sequence will be effective modulators of the relevant signal pathway.

The cPKC class of isozymes comprises the only members of the PKC general family that contains C2 regions. Other C2-containing proteins such as synaptotagmin and phospholipase Cγ also bind to a mixture of RACKs prepared from cell particulate fractions. It has also been demonstrated that recombinant fragments of synaptotagmin containing the C2 homologous region bind to mixtures of RACKs and inhibit PKC binding to RACKs (Mochly-Rosen, D. *et al.*, *Biochemistry* (1992) **31**:8120-8124).

The following experiments demonstrate that certain peptides residing in the C2 region of βPKC are able to inhibit translocation of βPKC and the maturation of *Xenopus* oocytes. Ron, D. *et al.*, *J Biol Chem* (1995) **270**:24180-24187.

The following βPKC-derived peptides were prepared:

βC2-1: KQKTKTIK (210-217);

βC2-2: MDPNGLSDPYVKL (186-198),

βC2-3 IPDPKSE (201-207);

βC2-4: SLNPEWNET (218-226);

Scrambled βC2-1: TKQKKITK,

Control Peptide: LQKAGVDG (266-271).

Recombinantly produced fragments of βPKC were expressed as fusion proteins with GST: Fusion L9 includes the V1 region, the pseudosubstrate sequence, and the C1 and V2 regions (residues 3-182) of βPKC. L10 includes the V1 region, the pseudosubstrate sequence and the first cysteine repeat from the C1 region, as well as the entire C2 and V3 regions (residues 3-76 and 143-339). The numbering is as described in Luo, J-H. *et al.*, *J Biol Chem* (1993) **248**:3715-3719.

Standard overlay assays were performed by blotting RACK1 onto nitrocellulose as described by Mochly-Rosen, D. *et al.*, *Proc Natl Acad Sci USA* (1991) **88**:3997-4000. Strips of the nitrocellulose sheet containing 0.1-1 μg RACK1 per strip were incubated in overlay buffer with or without the test fragment added at approximately 10 μM. Addition was in the presence or absence of 50 μg/ml phosphatidyl serine (PS) and 1 mM calcium. The mixture was further incubated for 30 min at room temperature. The strips were then washed and binding of fragment of L9 or L10 to RACK1 was detected with anti-GST polyclonal antibodies followed by labeling with anti-rabbit horseradish peroxidase-linked antibodies and development by addition of substrate.

Using this assay, L10, but not L9 was found to bind RACK1. The PKC activators phosphatidyl serine and calcium did not increase the binding of L10 to RACK1, although these activators are necessary for the binding of intact PKC to RACK1. Thus, these data are consistent with the suggestion that the PKC activators are required to expose the RACK binding site in the intact PKC; this site is already exposed in the C2-containing fragment L10.

To determine whether L10 would inhibit the binding of intact βPKC to RACK1, RACK1 was immobilized on an amylose column and βPKC binding in the presence of PS, DAG and calcium and in the presence of L10 or L9 was determined. In the presence of L10, βPKC binding to RACK1 was completely inhibited; however, this was not true of L9. Similar results were obtained in an overlay assay.

Similar overlay assays were conducted using the above-listed peptides as candidate inhibitors for the binding of L10 to RACK1. The C2-derived peptides

βC2-1, βC2-2 and βC2-4 peptides were successful in inhibiting binding of L10 to RACK1; however βC2-3 and scrambled βC2-1 were not.

In addition to the foregoing cell-free assays, the association of βPKC with RACK1 and the ability of peptides derived from the C2 region to interrupt this interaction was tested in rat neonatal cardiac myocytes in culture. The presence of RACK1 in these cells was confirmed by immunostaining. RACK1 was found at perinuclear structures and throughout the cytosol. Treating with NE or PMA did not alter these locations. It was also demonstrated that activated βII PKC, but not C2-less isoenzymes δ or εPKC, colocalized with RACK1

The C2-derived peptides that had been shown to inhibit βPKC binding to RACK1 *in vitro* were then tested for their ability to inhibit activation-induced translocation in myocytes.

The myocytes were exposed to 100 nM PMA for 15 min after transient permeabilization with saponin (50 μg/ml) in the presence and absence of the test peptides. 80% of the cells that had not been treated with peptides showed localization of β1 PKC to perinuclear structures. However, when βC2-1, βC2-2 or βC2-4 at 10 μM extracellular concentration had been supplied to the permeabilized cells, translocation of both βI PKC and βII PKC isoenzymes was inhibited by 65-95% βC2-4 was the most effective. Control peptides described above did not affect translocation.

Consistent with the results in Example 1, treating nonpermeabilized cardiac myocytes with 100 nM PMA resulted in translocation of εPKC from the nucleus to the perinuclear and cross-striated structures and of δPKC from the perinuclear and fibrilar cytosolic structures in 80% and 90% of the cells respectively. Permeabilization and treatment of the cells with the C2 peptides derived from βPKC had no effect on the translocation of these C2-less isozymes.

While the chronotropy of myocytes is not affected by βPKC isoenzymes, the insulin-induced maturation of *Xenopus* oocytes is mediated by the β form. Insulin treatment of these oocytes results in translocation of βPKC and maturation is delayed by the PKC-specific catalytic inhibitor pseudosubstrate peptide. PKC translocation is

blocked by injection of purified RACKs or a peptide corresponding to the PKC binding site on RACKs. (Smith, B.L. *et al.*, *Biochem Biophys Res Commun* (1992) **188**:1235-1240; Ron, D. *et al.*, *J Biol Chem* (1994) **269**:21395-21398).

Accordingly, the maturation of *Xenopus* oocytes was used as an alternative assay system to test the function of the peptides derived from the C2 region described above. In this assay, oocytes were injected with 50 μM of the test peptide one hour before insulin treatment (8.25 μg/ml). Insulin-induced oocyte maturation was then determined by monitoring the appearance of a white spot in the animal pole of the oocyte that is indicative of germinal vesicle breakdown in maturation. 10-15 oocytes were included per assay and oocytes were scored for 35 hours after treatment. As expected, βC2-1, βC2-2 and βC2-4 supplied in the range of 5 μM-500 μM significantly delayed oocyte maturation in a dose-dependent manner. The control peptides did not. The association of this effect with the prevention of translocation of βPKC to the particulate fraction in *Xenopus* oocytes was confirmed in a separate experiment. The peptide βC2-4 inhibited βPKC translocation but not θPKC in Jurkat T-cells. Figure 4 shows the effect of these various peptides on *Xenopus* oocyte maturation.

Example 3

Agonist Effect of Interacting Peptides

The oocyte maturation assay described above was also used to test the effect of various peptides derived from the PKC/RACK1 pair. Peptide I, derived from RACK1, as expected, inhibits the maturation of *Xenopus* oocytes presumably by interfering with the binding of βPKC1 to RACK1. On the other hand, a short peptide, rVI derived from the sixth WD-40 repeat in RACK1 enhances maturation, both in the presence and absence of insulin. Ron, D., Mochly-Rosen, D., *J. Biol. Chem.* (1994) **269**:21395-21398 These results are shown in Figures 5 and 5b. The rVI peptide is believed to interfere with the RACK-mimicking site on PKC which normally covers the RACK-binding site in the absence of activation.

Example 4

Interaction Peptides Derived from θPKC

θPKC is a member of the nPKC family and lacks a C2 region. Comparison of variable sequences with εPKC and other isozymes reveals regions of maximum disparity Of these regions, some are strongly conserved across vast phylogenetic spans, e.g., from mammals to the invertebrate *Aplysia*. Isozyme specific sequences that are strongly conserved by evolution are probable sites for binding cognate proteins. Comparing δPKC to θPKC in the analogous region allowed identification of a θ-specific peptide expected to interfere with PKC binding to a RACK. Peptides with these characteristics from the V1 region of θPKC were prepared and tested for their ability to inhibit the binding of θPKC V1 fragment to RACK1 *in vitro*. The results are shown in Figure 6 Of a multiplicity of peptides tested, both from other regions of the θPKC isoenzyme and from alternative isoenzymes in the family, only θV1 derived peptides T1 and T2, having the amino acid sequences GLSNFDCG (θPKC residues 8-15) and YVESENGQMYI (θPKC residues 36-46), respectively, were able to affect the interaction negatively. As expected, peptides rIII and rVI derived from the WD-40 regions of RACK1 were also effective.

The peptides T1 and T2 could inhibit the translocation of θPKC to the cellular particulate fraction in Jurkat T-cells. The specificity of this interaction is shown in Figure 11A which shows inhibition by the θPKC V1 peptide (T2) of the translocation of θPKC but not of βPKC.

Sequences derived from other PKC regions.

V3 region. The V3 or hinge region separates the regulatory and catalytic domains. This region contains the sites of proteolysis by trypsin and calpain. The lack of conservation of the V3 domain between the different PKC isozymes suggests that this section may also be at least, in part, involved in targeting the PKC isozymes to their anchoring proteins. Furthermore, it has been demonstrated that regions within the V3 of αPKC mediate the translocation of that isozyme to the nucleus (James G., and Olson E. *J. Cell Biol.* **116**:863-873, 1992). The V3 region of θPKC was found to

bind to RACK1 *in vitro*. Therefore, the V3 region could affect not only the targeting of the activated isozymes (in which the V3-region is exposed) but could also regulate the enzyme susceptibilities to proteolysis. Antibodies made against theta V3 fail to precipitate *fyn*, suggesting competition for the same sites on V3.

V5. The amino acid sequences of the βPKC isoforms βIPKC and βIIPKC are identical except for variability within the V5 region (35 amino acids for βIPKC and 38 amino acids for βIIPKC). Upon activation, βIPKC and βIIPKC translocate to different localization sites in the cell (Disatnik M.-H., Buraggi G., Mochly-Rosen D. *Exp. Cell Res.* (1994) **210**:287-297). This difference in localization of isozymes that are almost identical can be explained by the importance of the V5 region in mediating their targeting. Moreover, βIIPKC was found to selectively translocate to the nucleus upon proliferative stimulation where it selectively phosphorylated the nuclear envelope protein lamin B_1. (Murray N.R. *et al. J. Biol. Chem.* (1994) **269:**1385-2191).

PKC-related proteins. A distant homolog has been designated PKC-mu. It is unusual in that is has an N-terminal transmembrane domain. Recently the human cDNAs encoding two novel protein kinases have been cloned. These proteins termed PRK1 and PRK2 (protein kinase C related kinase 1 and 2) show high homology to each other and some homology to the regulatory region of PKC (Palmer R.H., *et al. Eur. J. Biochem.* (1995) **227**:344-351. Since the sequences within the regulatory domain of PKC are responsible for the interaction between a PKC and its anchoring proteins, sequences from PRK1 and PRK2 which show homology to functionally important sequences within the regulatory domain of PKC, are likely to be of biological importance.

Sequences from other isozymes and related proteins that meet the same isozyme selectivity/evolutionary conservation criteria include the following:

Peptides derived from the V1 region of PKC isozymes (Human)

Peptide	Sequence	Position
θV1-1	G-L-S-N-F-D-C-G	θPKC(8-15)
θV1-2	Y-V-E-S-E-N-G-Q-M-Y-I	θPKC(36-46)
θV1-3	I-V-K-G-K-N-V-D-L-I	θPKC(73-82)
θV1-4	D-M-N-E-F-E-T-E-G-F	θPKC(130-139)
δV1-1	A-F-N-S-Y-E-L-G-S	δPKC(8-16)
δV1-2	A-L-S-T-E-R-G-K-T-L-V	δPKC(35-45)
δV1-3	V-L-M-R-A-A-E-E-P-V	δPKC(72-82)
δV1-4	Q-S-M-R-S-E-D-E-A-K	δPKC(129-138)
εV1-1	N-G-L-L-K-I-K	εPKC(5-11)
εV1-2	E-A-V-S-L-K-P-T	εPKC(14-21)
εV1-3	L-A-V-F-H-D-A-P-I-G-Y	εPKC(81-91)
εV1-4	D-D-F-V-A-N-C-T-I	εPKC(92-100)
εV1-5	W-I-D-L-E-P-E-G-R-V	εPKC(116-125)
εV1-6	H-A-V-G-P-R-P-Q-T-F	εPKC(27-36)
εV1-7	N-G-S-R-H-F-E-D	εPKC(108-115)
ηV1-1	N-G-Y-L-R-V-R	ηPKC(9-15)
ηV1-2	E-A-V-G-L-Q-P-T	ηPKC(18-25)
ηV1-3	L-A-V-F-H-E-T-P-L-G-Y	ηPKC(84-94)
ηV1-4	D-F-V-A-N-C-T-L	ηPKC(95-102)
ηV1-5	W-V-D-L-E-P-E-G-K-V	ηPKC(120-129)
ηV1-6	H-S-L-F-K-K-G-H	ηPKC(31-38)
ηV1-7	T-G-A-S-D-T-F-E-G	ηPKC(111-119)
μV1-1	M-S-V-P-P-L-L-R-P	μPKC(1-9)
μV1-2	K-F-P-E-C-G-F-Y-G-L-Y	μPKC(86-96)
λV1-1	H-Q-V-R-V-K-A-Y-Y-R	λPKC(15-24)
λV1-2	Y-E-L-N-K-D-S-E-L-L-I	λPKC(87-94)
ζV1-1	V-R-L-K-A-H-Y	ζPKC(16-22)
ζV1-2	V-D-S-E-G-D	ζPKC(61-66)
ζV1-3	V-F-P-S-I-P-E-Q	ζPKC(95-102)

Peptides derived from the V3 region of PKC isozymes (Human):

Peptide	Sequence	Position
δV3-1	Q-G-F-E-K-K-T-G-V	δPKC(312-320)
δV3-2	D-N-N-G-T-Y-G-K-I	δPKC(327-335)
εV3-1	S-S-P-S-E-E-D-R-S	εPKC(336-344)
εV3-2	P-C-D-Q-E-I-K-E	εPKC(351-358)
εV3-3	E-N-N-I-R-K-A-L-S	εPKC(360-368)
εV3-4	G-E-V-R-Q-G-Q-A	εPKC(393-400)
λV3-1	M-D-Q-S-S-M-H-S-D-H-A-Q-T-V-I	λPKC(194-208)
λV3-2	L-D-Q-V-G-E-E	λPKC(218-224)
λV3-3	E-A-M-N-T-R-E-S-G	λPKC(227-234)
μV3-1	D-P-D-A-D-Q-E-D-S	μPKC(390-398)
μV3-2	S-K-D-T-L-R-K-R-H	μPKC(440-448)
μV3-3	I-T-L-F-Q-N-D-T-G	μPKC(457-465)
μV3-4	G-S-N-S-H-K-D-I-S	μPKC(559-567)
θV3-1	C-S-I-K-N-E-A-R-L	θPKC(322-330)
θV3-2	G-K-R-E-P-Q-G-I-S	θPKC(337-345)
θV3-3	D-E-V-D-K-M-C-H-L	θPKC(351-359)
ζV3-1	S-Q-E-P-P-V-D-D-K-N-E-D-A-D-L	ζPKC(194-208)
ζV3-2	I-K-D-D-S-E-D	ζPKC(217-223)
ζV3-3	P-V-I-D-G-M-D-G-I	ζPKC(226-234)
βV3-1	V-P-P-E-G-S-E-A	βPKC(290-297)
αV3-1	I-P-E-G-D-E-E-G	αPKC(290-297)
γV3-1	V-A-D-A-D-N-C-S	γPKC(290-297)

Peptides derived from the V5 region of PKC isozymes (Human)

Peptide	Sequence	Position
αV5-1	Q-L-V-I-A-N	αPKC(642-647)
βIV5-1	K-L-F-I-M-N	βIPKC(646-651)
βIIV5-1	Q-E-V-I-R-N	βIIPKC(645-650)
δV5-1	K-N-L-I-D-S	δPKC(649-654)
εV5-1	E-A-I-V-K-Q	εPKC(714-719)
ηV5-1	E-G-H-L-P-M	ηPKC(657-662)
λV5-1	D-D-I-V-R-K	λPKC(559-564)
μV5-1	S-D-S-P-E-A	μPKC(898-903)
θV5-1	R-A-L-I-N-S	θPKC(680-685)
ζV5-1	E-D-A-I-K-R	ζPKC(556-561)

Peptides derived from protein kinase C related proteins (Human)

Peptide	Sequence	Position
PRK1-1	Q-D-S-K-T-K-I-D	PRK1(171-178)
PRK2-1	Q-D-S-K-T-K-I-E	PRK2(181-188)
PRK1-2	E-L-A-V-F-W-R-D	PRK1(430-437)
PRK2-2	E-I-S-V-Y-W-R-D	PRK2(432-439)
PRK1-3	M-E-P-Q-G-C-L	PRK1(465-471)
PRK2-3	L-E-P-Q-G-T-L	PRK1(467-473)

μV1-1, μV1-2 derived from μPKC were picked because they aligned with εV1-2 and θV1-2 and part of θV1-1 respectively. λV1-1 and λV1-2 from λPKC were picked based on their alignment with εV1-2 and part of εV1-3 and θV1-2 respectively. ζV1-1, ζV1-2, ζV1-3 derived from ζPKC were picked according to their homology to εV1-2, θV1-2, and εV1-3 respectively. PRK1-1 and PRK2-2 were identified according to their homology to βC2-1. PRK1-2 and PRK2-2 were identified according to their homology to the biologically active εPKC-derived peptide εV1-3 and part of εV1-2. PRK1-3 and PRK2-3 were picked according to their alignment with the peptide εV1-5.

The peptide sequences were generated by aligning the human PKC sequences and the human PRK1 and PRK2 sequences using the MegAlign DNASTAR Inc. program. The sequences were aligned by using the clustal method. The algorithm groups sequences into clusters by examining the distances between all pairs. The clusters are aligned, first individually, then collectively to produce an overall alignment. (Higgins D.G. *et al.* (1989). *CABIOS* **5(2)**:151-153). The matrix for the alignment was PAM250 (percent accepted mutation 250-2.5 mutations per residue). This matrix allows only high stringency alignments.

Example 5

fyn as a PKC-theta Cognate

In this example, evidence is provided which identifies a specific protein as a cognate binding partner to PKC-theta in T-cells. This protein is a tyrosine protein kinase called *fyn*, which was previously known and believed to play an important role in T-cell function. Evidence is further provided to demonstrate that disrupting the localization of PKC-theta, using peptides from the PKC domain which interacts with *fyn*, depresses T-cell function.

The T-cell receptor (TCR) complex comprises at minimum the CD3 and CD4 complexes of proteins, to which several tyrosine kinases are associated. PLC-gamma, important in generating second messengers such as diacylglycerol and inositol triphosphate, is also a substrate for tyrosine phosphorylation. Among the non-receptor tyrosine kinases are ZAP-70 and the *src*-related proteins *fyn* and *lck*, believed to interact with CD3 and CD4 respectively; another tyrosine kinase, csk, is also associated with the TCR supercomplex of proteins.

In some reports, *fyn* is only included in 1% of the CD3 complexes. A transient association makes sense, however, in the context of what is known about other localization factors, such as RACK1 for PKC, which are similarly present in particular places in the cell only during particular signal transduction episodes. A role for *fyn* in T-cell signaling is well documented, including association with at least half a dozen other proteins which are also associated under some experimental conditions with TCR

(Penninger *et al., Immunol. Rev.* **135**:183-214 (1993). An association of *fyn* with PKC has not been previously observed.

The evidence that *fyn* interacts with PKC-theta comes from several independent and mutually supportive lines of experimentation. In most cases, the region of PKC-theta used to define the binding specificity was the V1 region (~140 amino acids), which is from the regulatory domain of PKC and is a sequence unique to this isozyme; some experiments also used the V3 domain, Figure 1. Proteins that interact with these regions fulfill the criteria defined in Examples 1-4. In the data provided in Example 1-4, RACK1 was shown to have some degree of binding to PKC-theta, which could be partially blocked with the T1 or T2 peptides (derived from the V1 region). Both peptides were also able to inhibit normal subcellular translocation of PKC-theta following treatment with activators of the signal transduction network; the latter experiments indicate the importance of the V1 region but do not suffice to identify the physiologically relevant cognate binding partner.

To identify the physiologically relevant cognate binding partner, a Triton (non-ionic detergent) cell extract was prepared from Jurkat T-cells (a human T-cell lymphoma line) using standard procedures. Based on the prior experience that physiologically relevant cognate binding partners for PKC may be associated with the particulate fraction, the "Triton extract" included both soluble and some particulate fraction proteins and is referred to herein as the Triton extract. A V1-*his* tail construct was also engineered; six histidine residues were attached to the N-terminus of V1. The six histidine residues bind to nickel agarose affinity beads.

Using these beads as an affinity extraction medium, the V1 region was immobilized and incubated with the Triton extract. After washing (by centrifugation in an Eppendorf tube), the bound proteins were eluted with strongly denaturing SDS gel sample buffer. After the eluate was separated by gel electrophoresis and transferred to a membrane, *fyn* was detectable at the correct molecular weight using a *fyn* specific antibody (Santa Cruz Biotechnology, Santa Cruz, CA). A band at the same molecular weight was also detected by antibodies to phosphotyrosine (Transduction

Laboratories, Lexington, KY). Antibodies to a related tyrosine kinase, *csk*, did not indicate any binding to the PKC domain.

Antibodies prepared against the V1/C1 boundary (isozyme-specific) region of PKC-theta were used to immunoprecipitate PKC-theta and any associated proteins from the particulate fraction of an extract from Jurkat T-cells (overnight at 4°). These associated proteins were separated by gel electrophoresis and blotted to a membrane. When the blot was probed with an antibody against phosphotyrosine, several bands were identified including a prominent one at the MW of *fyn*, 59 kDa, Figure 7A. By contrast, a band at the MW of *lck*, another *fyn*-related tyrosine kinase, was not identified as shown by probing with the appropriate antibody. Direct evidence that the coprecipitated protein is *fyn* was provided by staining with a commercially available labeled antibody prepared against *fyn*, Figure 7B. As a control for specificity, antibodies to PKC-beta were successfully used to coprecipitate RACK1, the known cognate binding protein for this isozyme; *fyn* was not coprecipitated in this case.

To define the *fyn* subsequence specificity of interaction, a modified yeast two hybrid system was used (U.S. Patent No. 5,283,173, Vojtek *et al. Cell* 74:205-214 (1993). Gene sequences for PKC-theta V1 and *fyn* were cloned as fusions to complementing halves of a transcription factor. In the particular system used, two reporter genes become activated as a consequence of the association between the two hybrid proteins, which thereby restores the holoenzyme status of the transcriptional activator. The first reporter is a histidine auxotroph repair enzyme, allowing growth selection; the second is beta-galactosidase, whose activity can be visualized with the substrate x-gal which turns blue following enzymatic cleavage. Visual inspection of the colonies allows scoring of interactions as strong (full color development within 2 hours), weak (by 12 hours), null (no signal at 24 hours). Liquid phase assays can also provide quantitative data. Miller, J.H. Experiments in Molecular Genetics (1972) Cold Spring Harbor Laboratory Press.

The catalytic domain and the regulatory domain of *fyn* were therefore tested separately as binding partners to the V1 region of PKC-theta. The catalytic domain gave a strong signal, and the regulatory domain gave a medium signal. It is not known

if each domain folds equally well into a stable structure, so this difference is not conclusive as to what portion of *fyn* has the best binding to V1. The V3 region of PKC-theta has also been tested against the *fyn* regulatory, and catalytic domain constructs; both interact as measured by *his* selection, with beta-galactosidase experiments all showing strong interaction. It thus appears that PKC-theta interacts with *fyn* over a substantial contact surface, in contrast to coprecipitation experiments using antibodies to the V1 region. In these experiments, antibodies to the V3 region failed to immunoprecipitate *fyn*, suggesting that the V3 antibodies and *fyn* are binding in part to the same site.

Figure 8 thus shows the results of the two-hybrid assay wherein the *fyn* peptide to be tested is fused to a DNA encoding the polymerase activating domains (of the VP16 transcription factor) and the relevant portion of the PKC-theta protein is fused to the DNA binding region of the transcription factor (LexA). Beta galactosidase was used as a reporter gene for the results in this Figure.

Figure 8A shows the constructs and Figure 8B summarizes the results. In addition to full-length *fyn*, the regulatory regions represented by *fyn* positions 1-259 interact strongly with the V1 and V3 regions of PKC-theta as does the kinase region from positions 259-534. *fyn*ΔSH2, representing positions 1-143 shows diminished interaction with both V1, although interaction is still present. A peptide representing positions 1-88 interacts only weakly with PKC-theta V3 and does not appear to interact with V1. Only very weak interaction between positions 88-143 (SH3) and 143-259 (SH2) with PKC-theta V1 is observed.

To test the specificity of the interaction of PKC-theta V1 with the *fyn* domains as compared to other proteins, the two hybrid system constructs incorporating the *fyn* portions were diluted into a large excess of random cDNA clones, prepared from murine T-cells, in the analogous vector. For the kinase domain, 10 of 12 positive clones picked at random were the *fyn* construct which had been spiked into the library; for the regulatory domain, 3 of 6 were from the spiked *fyn* construct. The other positive clones are further described in Example 8. The interaction of PKC-theta with

fyn appears to be specific since an analogous construct using the V1 region of the most closely related isozyme, PKC-delta, did not appear to bind to *fyn*.

Figure 9 represents a diagram and sequence of the *fyn* splice variant detected in this example, p59*fyn*. It is possible that PKC binds at the interface between the two *fyn* domains, which are both exposed upon activation. A PKC consensus phosphorylation site has been identified within the primary sequence of *fyn*, centered on threonine-297, in a short stretch of sequence that also scores high as part of an ATP binding consensus site. It is known that *fyn* has alternative splicing forms; the form found in T-cells includes the consensus PKC site, although this site is not unique to T-cells. Interestingly, addition of ATP to Jurkat T-cell extracts reduces the association of PKC-theta and *fyn*, measured by immunoprecipitation further suggesting a physiological interaction between the two proteins. By weaker criteria for a PKC consensus site, *fyn* has 13 additional potential phosphorylation sites. Finally, it is further possible that PKC is a substrate for *fyn*, since there are 5 tyrosine residues in the V1 region; tyrosine-36 looks particularly reasonable in this regard.

The first PKC cognate binding protein discovered, RACK1, was a clear member of the WD40 family of proteins, characterized by having multiple tandem copies of a sequence of ~40 amino acids with a conserved WD pair towards the C-terminus. *Fyn* also has three repeats with weak WD40 homology (aa 51-270), beginning in the middle of the Unique region and ending early in the catalytic domain. At a more detailed level, RACK1 shares several other short sequences of homology with *fyn*, both in its regulatory and catalytic domains.

Sequence variation between three alternatively spliced human *fyn* forms is found between amino acids 242-270 (the end of the regulatory domain and the beginning of the catalytic domain, which includes the N-terminal of the third WD-40 repeat). Interestingly, the WD-40 motif landmarks are conserved in all the alternative spliced forms, including S-253, KD-257-258, and WEV 260-262.

Since *fyn* has an SH3 domain, other homologs of which are known to bind proline rich domains, it is noteworthy that PKC-theta has a moderately praline rich region of ~50 residues (includes 10 prolines), accounting for a substantial part of the

V3 region. Antibodies to the V3 region failed to immunoprecipitate *fyn*, suggesting that the antibody and *fyn* are binding in part to the same site These results are not conclusive since the V3 region gave positive results in the two-hybrid system Most other PKC isozymes do not contain proline rich regions, including the most closely related isozyme PKC-delta. The only other isozyme in which a moderately proline-rich domain is found is PKC-mu, a recently described member of the class with less overall PKC homology

We have also observed that the T2 peptide, which blocks PKC-theta translocation as shown in Figure 10A and hence blocks association with the cognate localization factor, causes measurable suppression of IL-2 production from activated Jurkat T-cells, Figure 10B

In summary, the key independent lines of evidence indicating that *fyn* is a physiologically relevant binding partner for PKC-theta in T-cells are. (i) a PKC-theta variable domain pulls *fyn* out of a cell extract in an affinity binding mode; (ii) antibodies to PKC-theta variable domain immunoprecipitate a complex of PKC and *fyn* from cell extracts; (iii) PKC-theta variable domains and *fyn* interact in the yeast two hybrid system. In all cases, appropriate controls using closely related proteins show specificity of the interaction.

The existence of cognate binding proteins has been previously described, as have methods for their identification and their utility in drug discovery (for example see Fields, US Patent No. 5,283,173 and US Patent No. 5,352,660). The actual identification of *fyn* as a partner for PKC-theta can now be used in such art-known methods to identify and isolate compounds which block *fyn*/PKC-theta interactions. Such agents can be used to modulate biological activities which are mediated by *fyn*/PKC-theta binding; these include activities associated with the immune system

Example 6

Assay for Immune Modulating Compounds Using PKC-theta Translocation as an Index

To assess translocation, suitable cells, preferably nontransformed human T-cells, are cultured to a density of 10^6/ml and then incubated in cytokine-free medium for overnight. Ten-milliliter aliquots are used for each assay.

The substance to be tested is added to the appropriate samples and incubated for 15 minutes at 37°C. Substances known to stimulate the translocation of PKC-theta are then added: typically PMA at 20-80 nM ± PHA at 1 μg/ml are added, and the culture is incubated for 15 minutes at 37°C.

After the incubation period, the samples are spun at 1000 rpm for 10 minutes and the cell pellet is washed with cold PBS. The cells are then resuspended in homogenization buffer and sonicated. They are then centrifuged at 55000 rpm for 30 minutes to obtain a supernatant cytosolic fraction and a pellet which is resuspended in homogenization buffer with a 27-gauge needle to obtain the particulate fraction. After normalization of total protein, the content of PKC in each of the supernatant and particulate fraction is then determined using SDS-PAGE and detection using appropriate antibodies.

Figure 11 shows the results of SDS-PAGE of the soluble and particulate fraction stained with anti-PKC-theta antibodies (Figure 12A) or anti-PKC-beta antibodies (Figure 12B). As shown, PKC-beta is essentially all in the soluble fraction in unstimulated cells, while PKC-theta is more or less evenly distributed between these two fractions. Presumably, this is the case because proliferation of these cells requires the presence of added cytokines, which may effect partial activation of the T-cells. When deprived of cytokines in the medium for, for example, overnight, the levels of PKC-theta in the soluble fraction are increased. In any case, after stimulation with either PMA or a combination of PMA/PHA, which directly cause PKC activation, both PKC-theta and PKC-beta are translocated to the particulate fraction. However, stimulation with OKT-3, an antibody immunoreactive with the CD3 component of T-cell receptors, which provides a more focused physiologically based activation,

results in translocation of PKC-theta, but not PKC-beta. The ability of OKT-3 to effect, specifically, translocation of PKC-theta is further evidence of the involvement of PKC-theta in transduction of T-cell receptor mediated stimulation.

The use of the foregoing as an assay for identifying an immune system modulator is shown in Figure 12. In the assay conducted as described above, the cells were initially incubated with 20 μM of a candidate compound TER14687. As shown in Figure 12, TER14687 was able to inhibit the translocation of PKC-theta to the particulate fraction when either OKT-3 or PMA/PHA was used as a stimulant. (The assay performed with OKT-3 involved one hour of incubation with OKT-3, rather than the 15-minute period described above.)

TER14687 was also shown to increase the proportion of PKC-theta in the soluble fraction in resting T-cells. T-cells are generally grown in the presence of exogenously added cytokines and are thus in a partially activated state. When cytokine stimulation is withdrawn for a period of time, PKC-theta concentration in the soluble fraction normally increases. TER14687 thus facilitates decay to the resting state.

The effect of TER14687 on the PKC-theta/cognate interaction can also be demonstrated by showing that TER14687 prevents tyrosine phosphorylation of a 21 kD protein after OKT-3 stimulation of T-cells, in which the foregoing protocol was followed except that phosphorylation was measured directly, with an Ab to phosphotyrosine. This phosphorylation is a known downstream event of T-cell activation.

Example 7

Assay for Modulation Indexed by Binding

To visualize endogenous PKC cognates T-cell proteins are separated by SDS gel electrophoresis and transferred to nitrocellulose membranes. A large number of proteins are thus distributed on the nitrocellulose. The nitrocellulose is then incubated with PKC-theta and washed, and the bound PKC-theta is visualized using labeled anti-PKC-theta. PKC-theta protein was produced Sf9 insect cells using a baculovirus

expression system, Invitrogen, San Diego, CA. In the absence of substances known to activate PKC, essentially no bands appear. In the presence of PKC activators, a large number of bands is seen. Some of these bands may be true endogenous cognates; others may mimic cognate binding abilities in the denatured state characteristic of proteins transferred to nitrocellulose membranes. As such, these proteins are substitutes for the endogenous cognates.

In the presence of TER14687 during PKC-theta incubation on the blot, binding is diminished, thus showing the modulating effect of TER14687 on cognate binding.

In addition to TER14687, the following peptides derived from the regulatory region of *fyn* were tested: *fyn2*, representing residues 111-118 underlined in Figure 9, showed partial inhibition of PKC-theta binding to *fyn*; and *fyn3*, residues 188-195 underlined in Figure 9, showed complete inhibition of PKC binding. *fyn2* and *fyn3* are derived from the portion of *fyn* which shows homology to WD40 repeats. An additional peptide, *fyn1* was not capable of inhibiting binding; this peptide was derived from a *fyn* splice variant different from that shown in Figure 9. Another inactive peptide was *fyn4*, representing residues 441-449 from the catalytic domain.

Example 8

Additional Cognates Identified by the Yeast Two-Hybrid System

The intracellular method to detect peptide/peptide binding, described in Example 5 hereinabove was used a) to find additional peptides which bind to PKC-theta and b) to provide an assay system for the effect of candidate substances on interaction with PKC-theta and its cognate.

First, the yeast two-hybrid system was used to retrieve cDNAs encoding peptides that interact with PKC-theta.

In this approach, plasmids containing the polymerase activating domain fused to the kinase or regulatory domains of *fyn* were mixed with similar vectors containing a cDNA library from the murine T-cell line HT2 in a ratio of 1:500. Yeast harboring plasmids containing PKC-theta V1 fused to the DNA binding domain were

transformed with this mixture of plasmids at one-tenth of the regular protocol. The yeast were plated onto media selecting for presence of the plasmids (THULL) plates and assayed for β-galactosidase on filter lifts. Positive colonies were picked on THULL grids and retested. DNA was extracted from colonies which remained positive, and then amplified using 5' and 3' plasmid-based polylinker flanking primers and the products were analyzed by Southern blot using *fyn* as a probe.

Of 12 positives found when *fyn* kinase domain was mixed into the cDNA library, 10 were the *fyn* kinase itself. However, two contained sequences other than those derived from *fyn*.

Of six positives obtained from the mixture when the cDNA library had been mixed with the *fyn* regulatory domain, three contained DNA sequences other than *fyn*. Two of these, designated 2-10 and 2-40 have identical nucleotide sequences not yet identified with any known sequence and the other, 2-32 appears to be a mouse homolog of a recently isolated human gene, ELL. The cDNA insert in 2-10 and 2-40 contained 1083 base pairs with an open reading frame encoding a previously unknown 335 amino acid peptide with several potential zinc-finger motifs. The cDNA hybridizes with a 2.4 kD transcript present in lymphoid tissue and a 6 kD transcript present in heart and skeletal muscle as shown by Northern blot.

Both clones 2-10 and 2-32, when tested in a liquid β-gal assay, were able to interact with PKC-theta V1 in the two-hybrid system described above. When transformed into the parental yeast strain L40, lacking PKC-theta V1, no activation was observed by a reporter assay, nor was activation observed when theta V1 was replaced by delta V1 or an unrelated protein, lamin.

Using the same assay, the effect of TER14687 on the interaction of PKC-theta V1 with either *fyn* or with clones 2-10 and 2-32 was tested. In this assay, yeast containing the above-described two-hybrid system were inoculated 1:10 dilutions into 1 ml THULL containing 40 μM TER14687 or a control compound in DMSO. Various other controls were included; for example, TER14687 did not inhibit expression of the reporter gene when the transcription factor (LexA/E2A) was supplied in covalently bound form. The activity of β-galactosidase produced was

measured as OD at 420 nm. TER14687 markedly diminished binding theta V1 to *fyn* or the substitute cognates generated from 2-10 and 2-32, as shown in Figure 13.

In addition, several clones encoding apparent cognate proteins were obtained from a human's $CD4^+$ T-cell cDNA library using the same yeast two-hybrid protocol. The cDNA library was constructed using T-cells isolated from donor blood and had a complexity estimated between 10^5 to 5×10^6 independent clones. Out of 1.4×10^7 clones screened, 63 remained positive after a secondary βGAL filterlift assay.

Thirty-nine of the clones appear to have identical sequence and the same approximate cDNA insert size. The sequence contains an open reading frame and is clearly a partial sequence which does not match any data base sequence. One of these clones, No. 10, was retested in the yeast two-hybrid system and found to bind θV1, but not δV1 or lamin.

In addition, two clones from this library, 1-22 and 1-23 share sequence with human elongation factor 1-γ. 1-23 overlaps with 1-22, but both are partial sequences. Both bind specifically to θV1 in the yeast two-hybrid assay.

One additional clone, 2-18 also shows specific binding to θV1 in the yeast two-hybrid assay and shows high homology with SH3-containing c-abl binding proteins which inhibit c-abl transformation. U17698 murine abl philin-1 is 89% homologous; U31089 human Abl binding protein 3 is 94% homologous; and U23435 human Abl interactor 2 is 88% homologous.

Two additional clones were obtained in a similar manner. Clone 2-20 contains 729 bp as a partial cDNA matched to human elongation factor 2. Clone 3-1 contains 839 bp with high homology to HSV1 transducing factor α.

The nucleotide sequences and the amino acid sequences encoded for clones 2-10, 2-32, #10, 1-22, 2-18, 2-20 and 3-1 are shown in Figure 15.

Example 9

Association of PKC-theta Activation with Allergic Reactions

A human T-cell line, TT7.5, is physiologically activated with OKT3 coated on tissue culture plates at 10 μg/ml as shown by enhanced proliferation using a tritiated thymidine assay. Activation is also characterized by enhanced secretion of interferon-γ, and of interleukins 4 and 5, but not of IL-2, as assayed in appropriate ELISAs. This pattern characterizes a Th2-like cell. Th2 cells have been shown to mediate allergy via the immunoregulatory effects of IL-4 and IL-5 and can thus be used as an *in vitro* model of T-cell function in mediating allergy.

As was shown above, OKT3 stimulation of T-cell lines, including TT7.5 cells, results in translocation of PKC-theta. In a similar assay that uses cytokine production as a measure of PKC-theta interaction with cognate, TER14687, administered during OKT3 activation, inhibits the production of IL-4 and IL-5, but not the induced production of interferon-γ, as shown in Figure 14. This pattern of cytokine production is associated with reduced stimulation of IgE and eosinophil production. Similar results are obtained with the Th2-like T-cell line TT3.6. In a Th1-type cell line (TT6.4) TER14687 inhibits OKT3-induced IL-2 production, but not interferon-γ production.

TER14687 also strongly inhibits expression, in TT7.5 cells, of the surface markers of T-cell activation, such as CD69, CD25 and CD40L following overnight OKT3 stimulation. CD69 is expressed exclusively on activated T-cells, CD25 is a low-affinity IL-2 receptor expressed on activated T-cells and CD40L is a ligand for CD40 which is also expressed exclusively on activated T-cells. These markers were assayed using appropriately labeled fluorescent antibodies and a flow cytometer.

Similar results were obtained in Jurkat cells following activation by PMA rather than OKT3. In the presence of 50 μM *fyn2* peptide, enhanced expression of CD69 is reduced.

Claims

1. A method to identify a modulator of intracellular signal transduction mediated by a signal-generating protein from a library of candidate molecules, which method comprises

providing an environment containing said signal generating protein or a peptide derived therefrom and a partner cognate which binds specifically to the catalytically active signal-generating protein via a noncatalytic site of said signal-generating protein or a said cognate-derived peptide;

adding a candidate substance to said environment;

determining the interaction of signal generating protein or derived peptide with cognate or derived peptide in the presence and absence of said candidate;

comparing said interaction in presence and absence of candidate;

wherein a candidate which modulates said interaction is identified as a modulator of intracellular signal transduction, and

wherein said determining is performed under conditions not dependent on purity of the cognate or signal generating protein.

2. The method of claim 1 wherein said partner cognate is identified by a method which comprises

(a) preparing an extract of intracellular components and/or membrane bound receptors;

(b) separating said intracellular components and/or membrane bound receptors on a solid support to obtain a multiplicity of said components and/or receptors in a multiplicity of locations on said support;

(c) probing said solid support with the signal-generating protein or a fragment thereof; and

(d) identifying at least one component or receptor on the solid support which binds said signal-generating protein, thus identifying said cognate.

3 The method of claim 2 wherein said intracellular components and/or membrane bound receptors are subjected to proteolysis prior to the separating of step (b); and/or

wherein said signal-generating protein or fragment is radioactively labeled, labeled with a fluorescent label, or labeled with a biotin label, or is detected by means of an antibody or fragment thereof.

4 The method of any of claims 1-3 wherein said interaction results in a metabolic effect selected from

maturation of a *Xenopus* oocyte;

induction of negative chronotropy in cardiac myocytes by an inducer;

transcription of a reporter gene; and

subcellular translocation of a catalytically active signal-generating protein

5 The method of claim 1 which comprises

providing, in recombinant host cells,

a first expression system which comprises a nucleotide sequence encoding said signal-generating protein or a peptide derived therefrom in reading frame with nucleotide sequence encoding a first portion of a reporter protein, wherein said first portion does not itself possess the detectable property of the reporter protein, and

a second expression system which comprises a nucleotide sequence encoding a second portion of said reporter protein, wherein, when said first and second portions of the reporter protein are in physical proximity, the reporter protein property is exhibited, in reading frame with a nucleotide sequence encoding said cognate or cognate-derived peptide; and

co-expressing said first and second expression systems, whereby expression of said cognate or cognate-derived peptide that binds to the signal-generating protein or peptide derived therefrom results in display of the properties of the reporter protein, in the presence and absence of the candidate molecules of said library, and

measuring the level of the reporter protein in the presence and absence of each of the candidate molecules of the library;

comparing the level of the reporter protein in the presence and absence of each of the candidate molecules of the library,

wherein an increase or decrease in the level of reporter protein in the presence as opposed to absence of the candidate molecule identifies the candidate molecule as said modulator.

6. The method of any of claims 1-5 wherein the signal-generating protein is a PKC isoenzyme

7 The method of claim 6 wherein the signal-generating protein is PKC-theta.

8 The method of claim 7 wherein the cognate is *fyn*.

9. The method of claim 6 wherein the peptide derived from the signal generating protein is selected from the group consisting of βC2-1, βC2-2, βC2-4, θV1-1, θV1-2, θV1-3, θV1-4, δV1-1, δV1-2, δV1-3, δV1-4, εV1-1, εV1-2, εV1-3, εV1-4, εV1-5, εV1-6, εV1-7, ηV1-1, ηV1-2, ηV1-3, ηV1-4, ηV1-5, ηV1-6, ηV1-7, μV1-1, μV1-2, λV1-1, λV1-2, ζV1-1, ζV1-2 and ζV1-3.

10. The peptide βC2-1, βC2-2, βC2-4, εPKCV1, θV1-1, θV1-2, θV1-3, θV1-4, δV1-1, δV1-2, δV1-3, θV1-4, εV1-1, εV1-2, εV1-3, εV1-4, εV1-5, εV1-6,

εV1-7, ηV1-1, ηV1-2, ηV1-3, ηV1-4, ηV1-5, ηV1-6, ηV1-7, μV1-1, μV1-2, λV1-1, λV1-2, ζV1-1, ζV1-2 or ζV1-3

11. A method to identify a substance that has immunomodulating activity which method comprises

providing an environment containing PKC-theta or a fragment thereof and its cognate or cognate-derived peptide under conditions wherein said PKC-theta or fragment interacts with said cognate or cognate-derived peptide;

adding a candidate substance to said environment;

determining the interaction of PKC-theta or fragment with cognate or cognate-derived peptide in the presence and absence of said candidate;

comparing said interaction in the presence and absence of candidate;

wherein a candidate which modulates said interaction is identified as having immunomodulating activity.

12. The method of claim 11 wherein said environment is an intracellular environment.

13. The method of claim 12 wherein determining said interaction is by

measuring translocation of said PKC-theta or fragment, or

measuring tyrosine phosphorylation of a 21 kD protein after OKT-3 stimulation of T-cells, or

measuring diminution of IL4 and/or IL5 production without affecting IFNγ production.

14. The method of any of claims 11-13 wherein said cognate or cognate-derived peptide is selected from the group consisting of *fyn*, the *fyn* fragment *fyn-3*, the *fyn* fragment *fyn-2*, and the protein encoded by clone 2-10 or 2-32 as described herein.

15. A method to modulate the immune system in a subject, said method comprising the step of administering to said subject an effective amount of a substance identified by the method of any of claims 11-14.

16. The method of claim 15, wherein said substance is administered to reduce T-cell activity in a subject in need of such reduction., or

wherein said substance is administered to reduce the rate of graft rejection in a subject in need of such reduction, or

wherein said substance is administered to reduce the severity of an autoimmune disorder in a subject in need of such reduction, or

wherein said substance is administered to ameliorate an allergic and/or asthmatic response in a subject in need of such amelioration, or

wherein said substance is administered to diminish cytokine production in a subject in need of such diminution.

17. A pharmaceutical composition comprising a substance identified by the method of any of claims 11-14 and a pharmaceutically acceptable excipient.

18. An isolated and purified protein cognate of PKC-theta encoded by the clone 2-10, 2-32, #10, 1-22, 2-18, 2-20 or 3-1.

19. An isolated nucleic acid molecule that encodes the cognate of claim 18.

20. A nucleic acid molecule which comprises an expression system which expression system comprises a nucleotide sequence encoding the cognate of claim 18 operably linked to control sequences for effecting its expression.

21. A recombinant host cell containing the nucleic acid molecule of claim 20

22 A method to produce a protein cognate of PKC-theta encoded by the clone 2-10, 2-32, #10, 1-22, 2-18, 2-20 or 3-1, which method comprises culturing the cells of claim 21 under conditions wherein said cognate is produced.

23. Antibodies immunoreactive with the protein cognate of claim 18.

24 An isolated nucleic acid molecule comprising a sequence complementary to the coding sequence contained in clone 2-10, 2-32, #10, 1-22, 2-18, 2-20 or 3-1

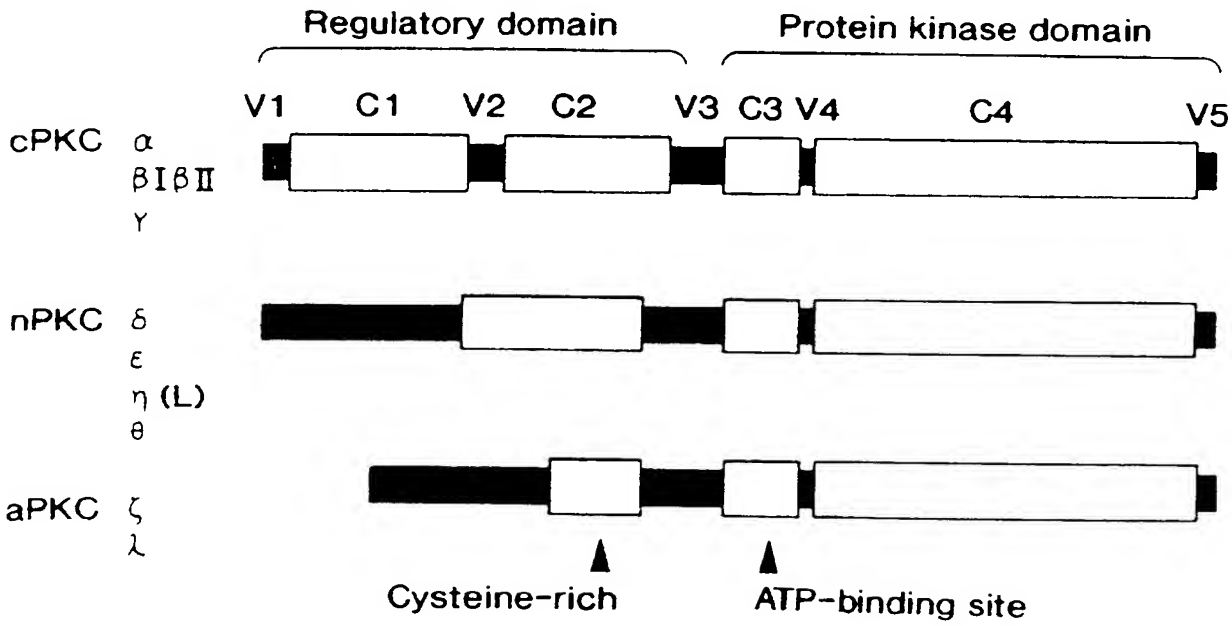

Regulatory domain
Protein kinase domain
V1
C1
V2
C2
V3
C3
V4
C4
V5
cPKC
α
βIβII
γ
nPKC
δ
ε
η (L)
θ
aPKC
ζ
λ
Cysteine-rich
ATP-binding site

FIG. 1

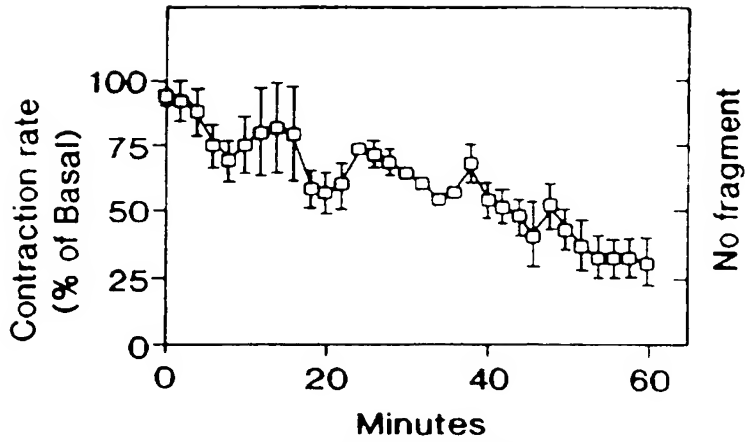


FIG. 2A

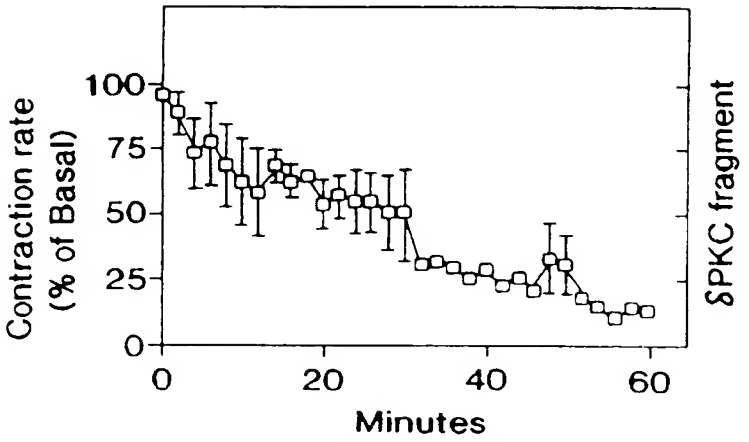


FIG. 2B

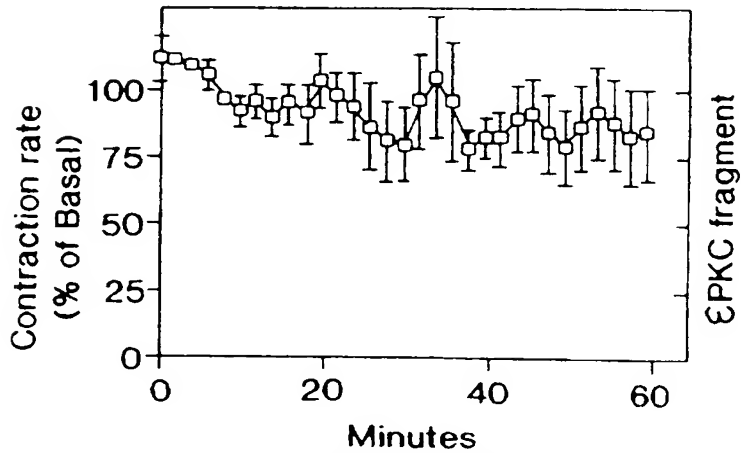


FIG. 2C

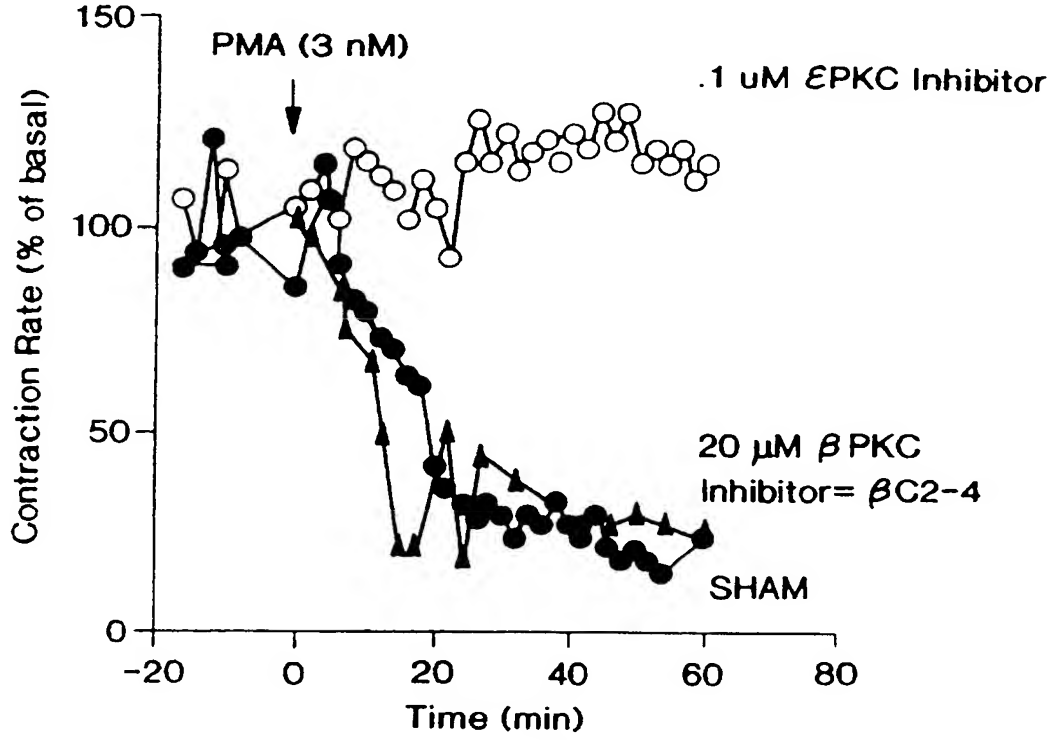

150
100
50
0
Contraction Rate (% of basal)
PMA (3 nM)
.1 uM εPKC Inhibitor
20 μM βPKC
Inhibitor= βC2-4
SHAM
-20
0
20
40
60
80
Time (min)

FIG. 3

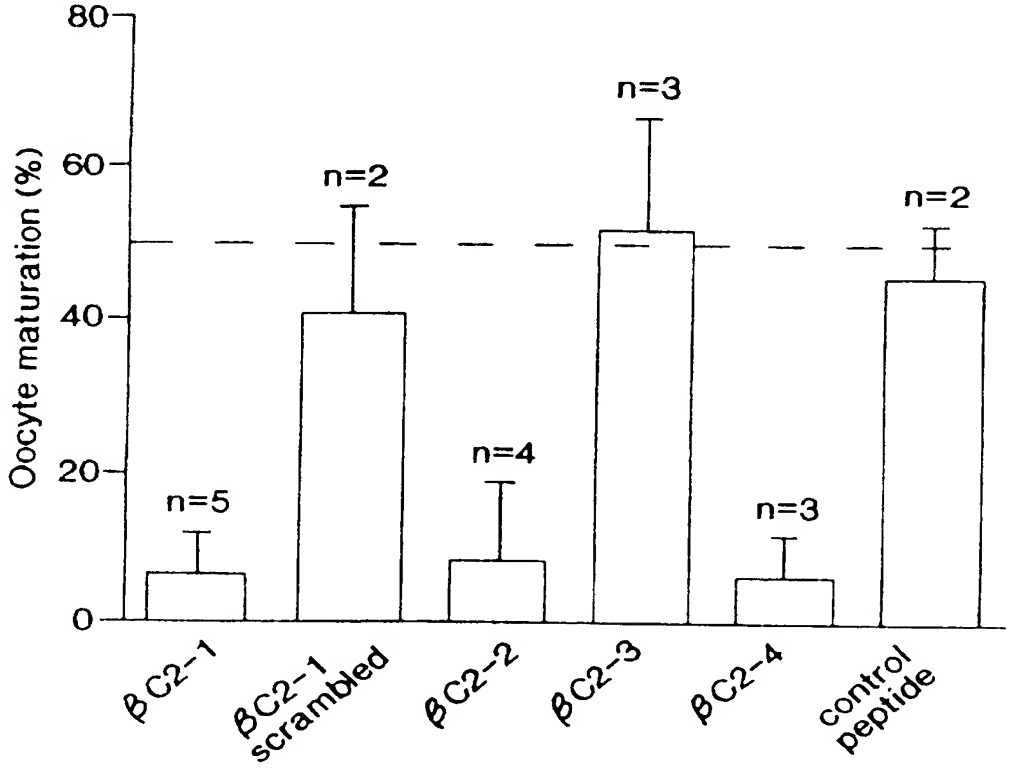

80
60
40
20
0
Oocyte maturation (%)
n=5
n=2
n=4
n=3
n=3
n=2
βC2-1
βC2-1 scrambled
βC2-2
βC2-3
βC2-4
control peptide

FIG. 4

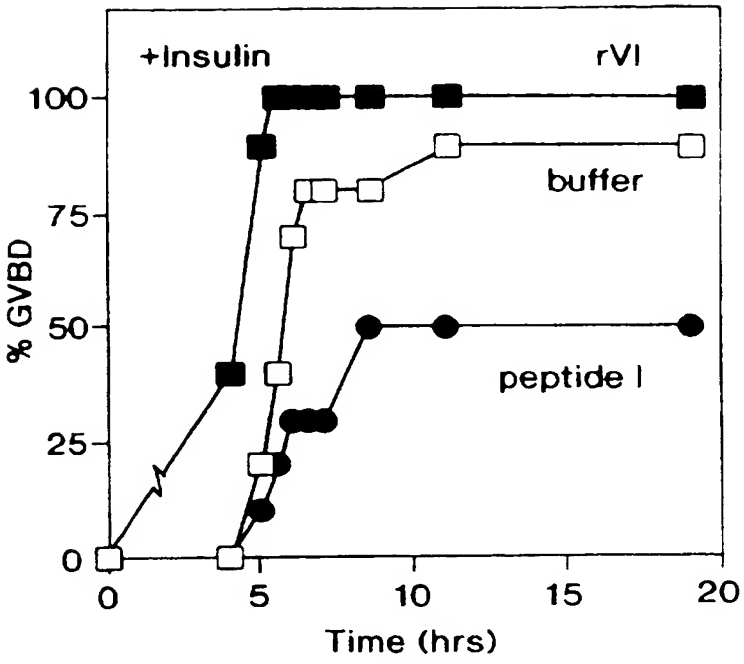

+Insulin
rVI
buffer
peptide I
% GVBD
100
75
50
25
0
0
5
10
15
20
Time (hrs)

FIG. 5A

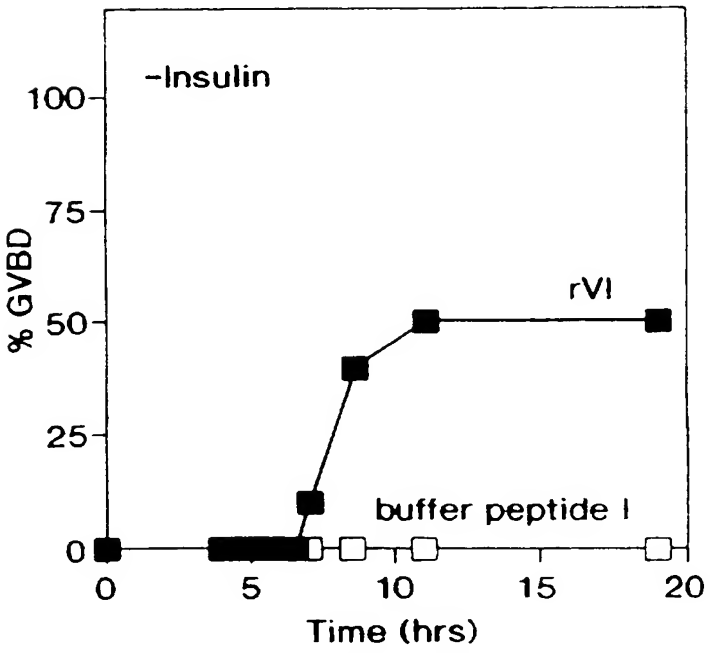

-Insulin
rVI
buffer peptide I
% GVBD
100
75
50
25
0
0
5
10
15
20
Time (hrs)

FIG. 5B

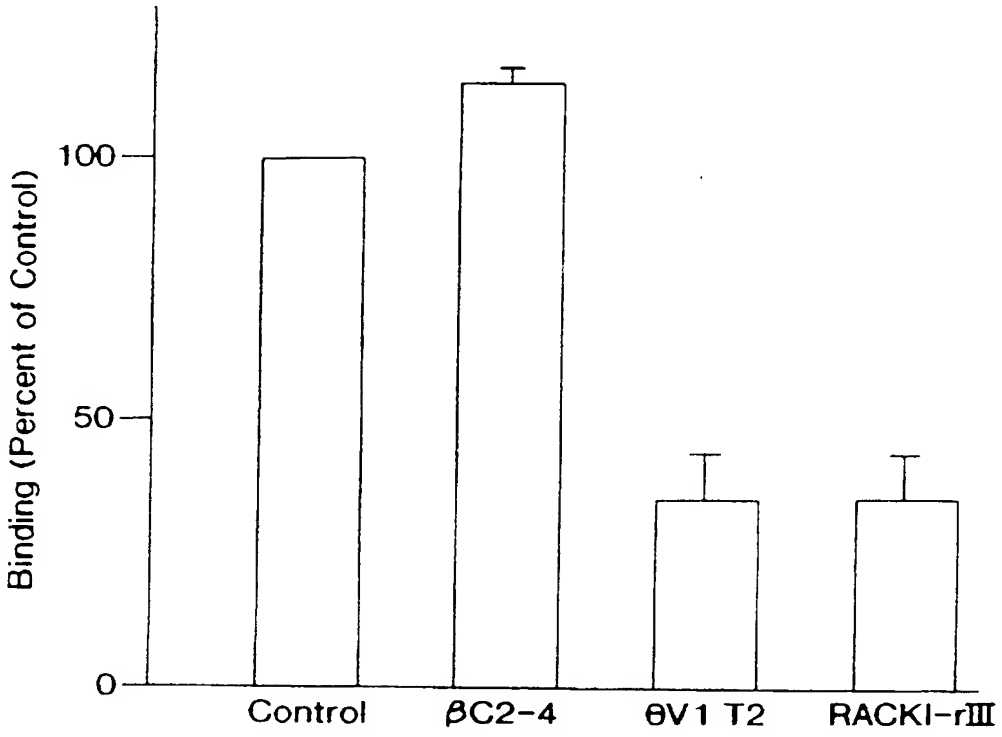

Binding (Percent of Control)
100
50
0
Control
βC2-4
θV1 T2
RACKI-rIII

FIG. 6

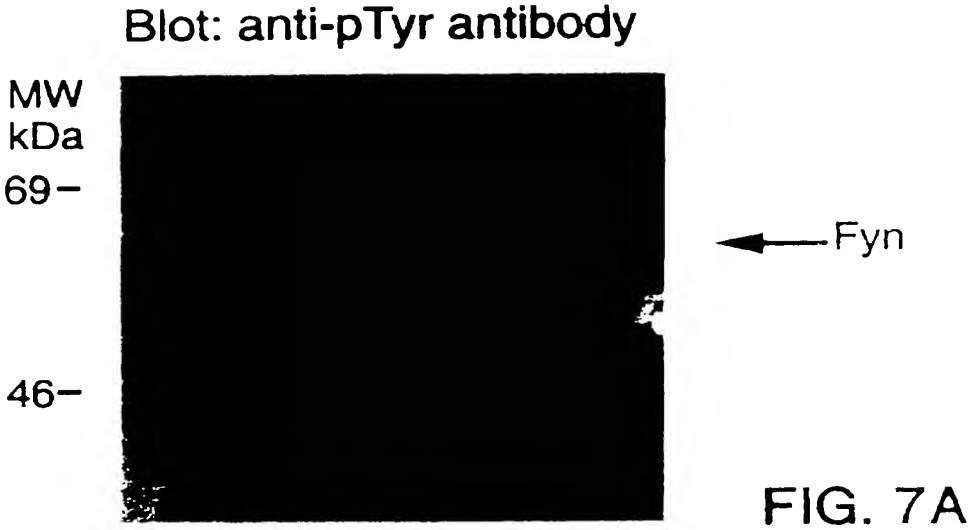

Blot: anti-pTyr antibody
MW
kDa
69–
46–
Fyn

FIG. 7A

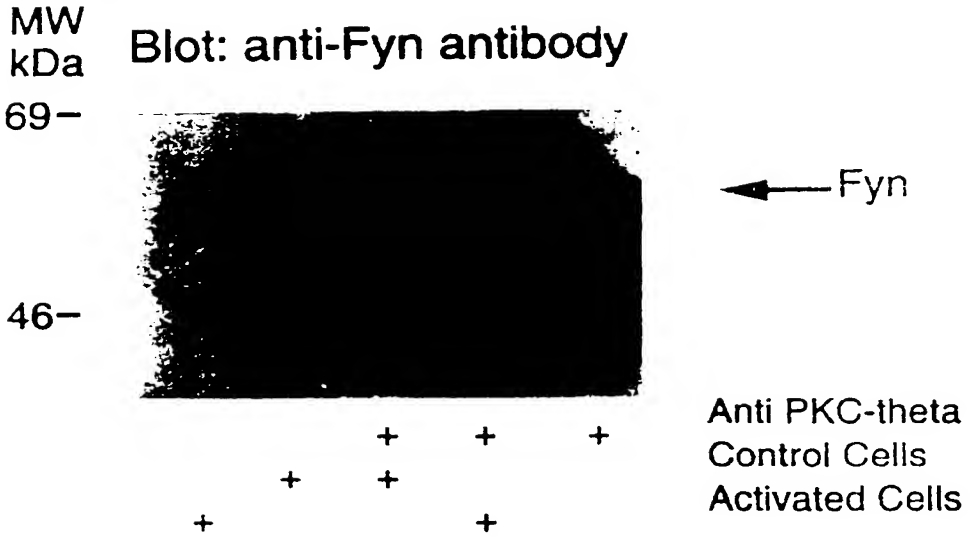

MW
kDa
Blot: anti-Fyn antibody
69–
46–
Fyn
+ + +
+ +
+ +
Anti PKC-theta
Control Cells
Activated Cells

FIG. 7B

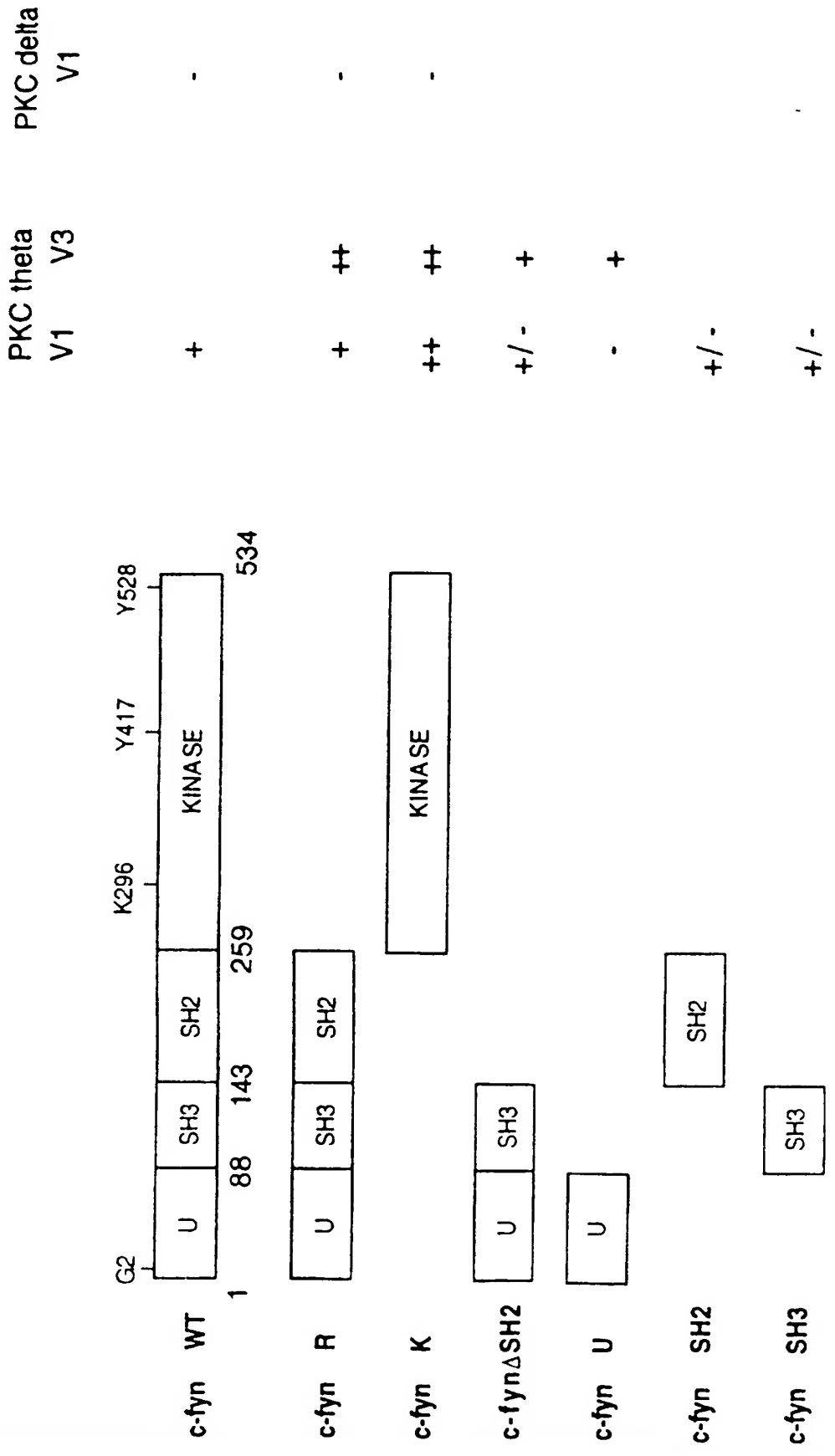

PKC delta
V1
PKC theta
V1 V3
c-fyn WT
c-fyn R
c-fyn K
c-fyn ΔSH2
c-fyn U
c-fyn SH2
c-fyn SH3
+
+
++
++
+/-
-
+/-
+/-
-
-
+
+
G2
1
U
88
SH3
143
SH2
259
K296
Y417
KINASE
Y528
534

FIG. 8

p59 Fyn

1 mgcvqckdke aaklteerdg slnqssgyry gtdptpqhyp sfgvtsipny 50

fyn2

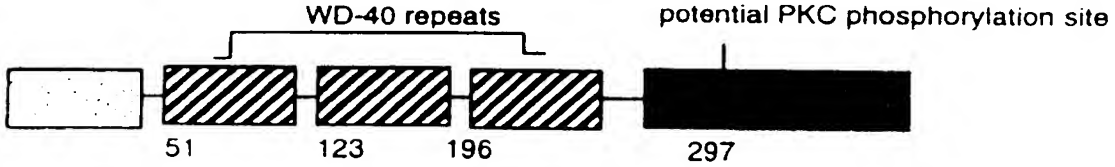
51 nnfhaaggqg ltvfggvnss shtgtlrtrg gtgvtlfval ydyartedd lsfhkgekfq ilnssegdwwea 122

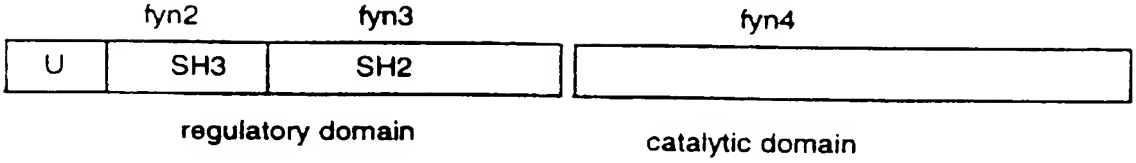
fyn3

123 rslttgetgyipsnyvapvdsiqaeewyfgklgrk daerqllsfgnprgtfliresqt tkgaysislsird wddm 195

196 kgdhvkhykirkldnggyyittraqetlqqlvq hysekadglcfnltvjassctpqtsglakdawevarrslcl 270

ekklgqgcfa evwlgtwngn tkvaiktlkpgtmspesfle eaqimkklkh dklvqlyavv seepiyivte
ymskgsllDflkdgegralk lpnlvdmaaq vaagmayier mnyihrdlrs anilvgnglickiadfglar liedneytar
fyn4
qgakfpikwt apeaalygrf tiksdvwsfgilltelvtkg rvpypgmnnr evleqvergymmpcpqdcpislhelmihc
wkkdpeerptf eylqgfledy ftatepqyqp genl





KTLK (bold,underline) = PKC phosphorylation site
underline = RACK1-homologues sequences
Box = WD-40 - like domain
1-14 myrstylation site
14-88 Unique region
88-143 SH3
150-233 SH2
250-516 catalytic domain

FIG. 9

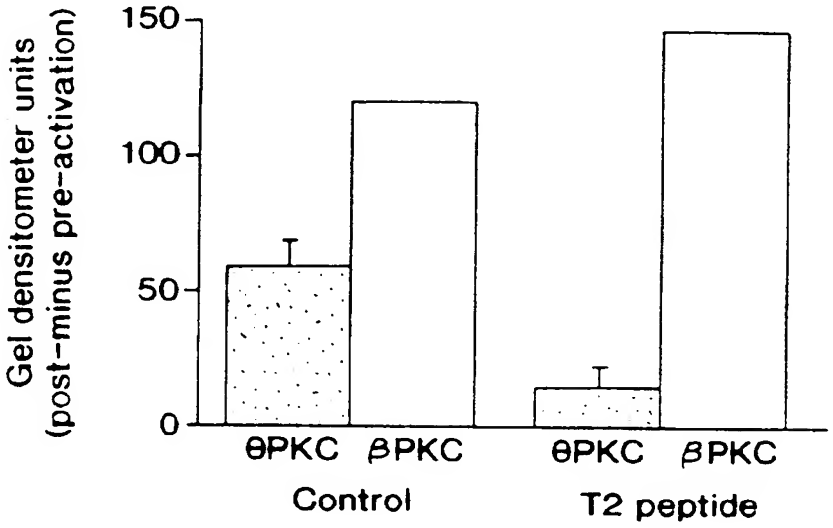


FIG. 10A

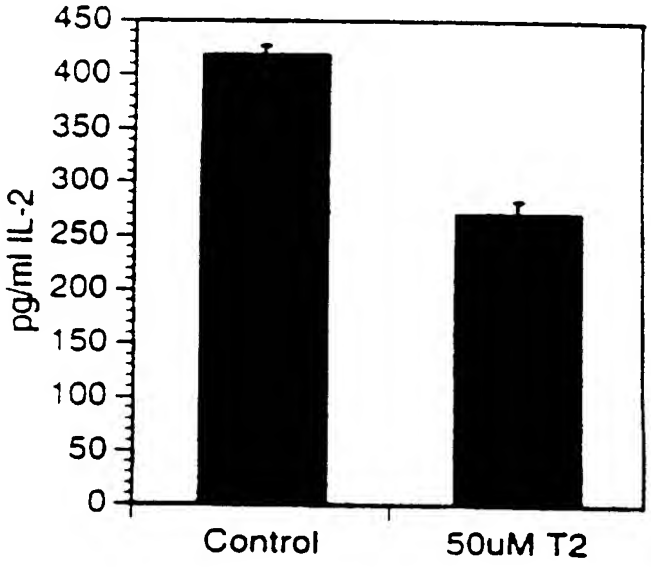


FIG. 10B

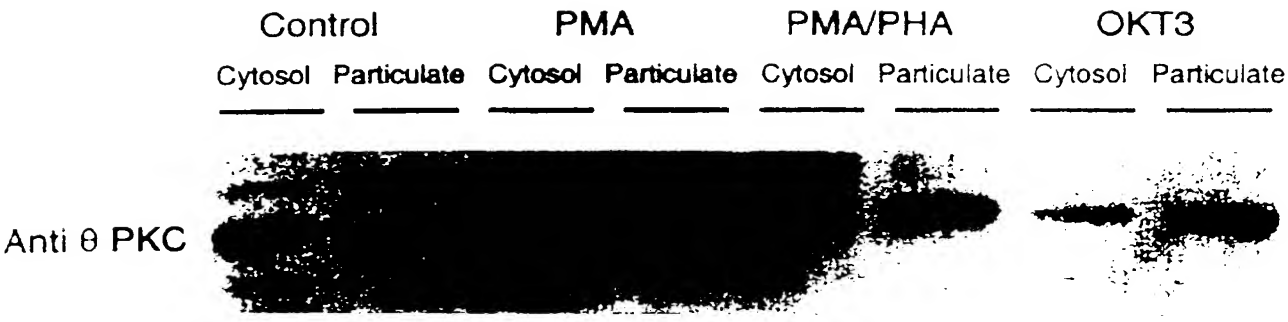


FIG. 11A

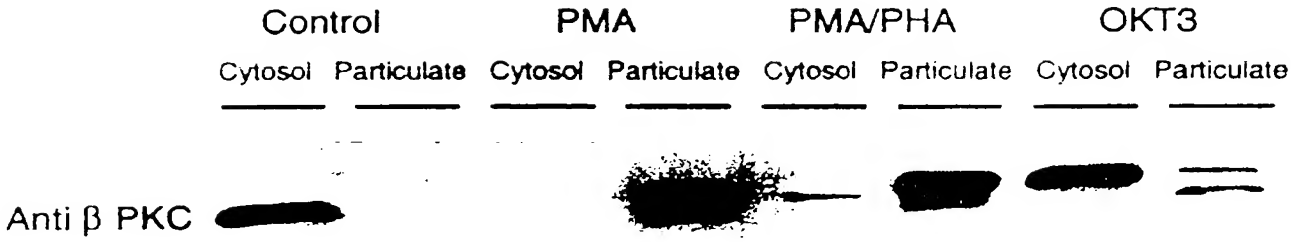


FIG. 11B

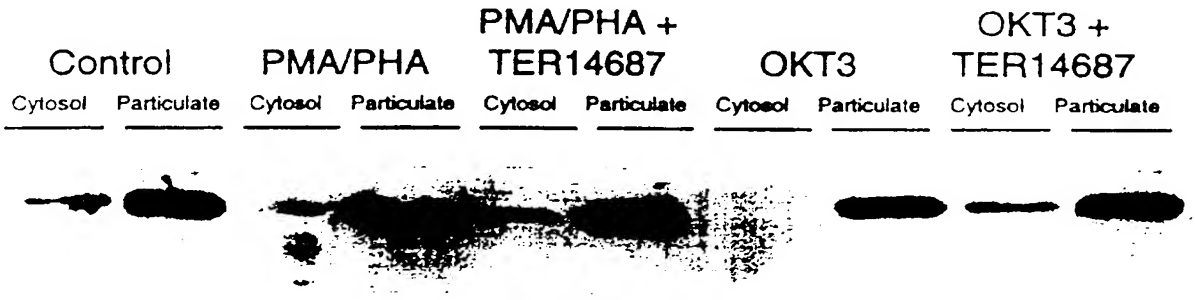

Control
PMA/PHA
PMA/PHA + TER14687
OKT3
OKT3 + TER14687
Cytosol
Particulate
Cytosol
Particulate
Cytosol
Particulate
Cytosol
Particulate
Cytosol
Particulate

FIG. 12

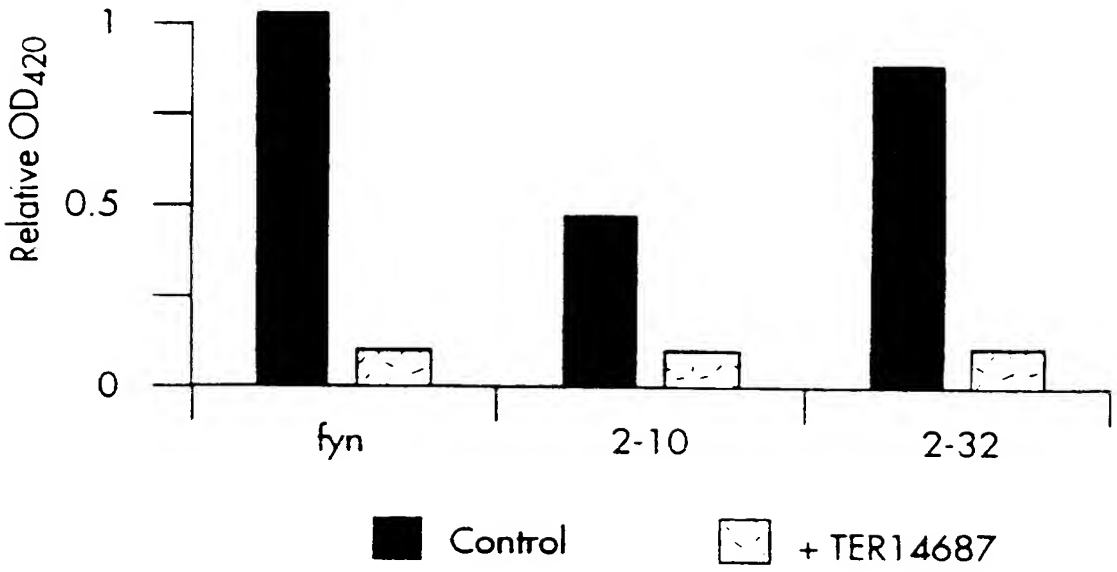
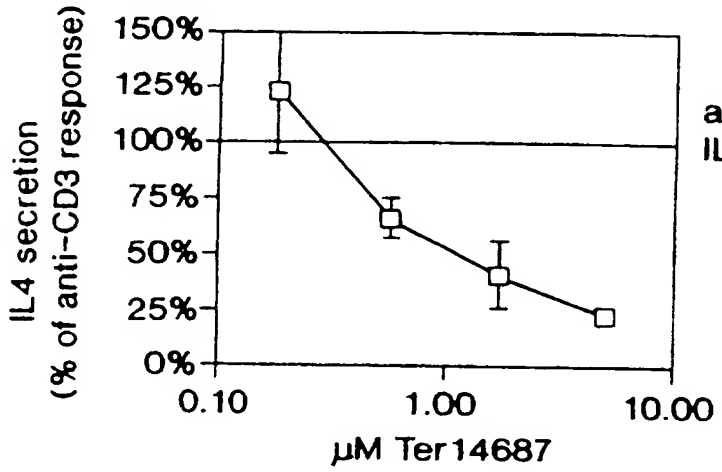
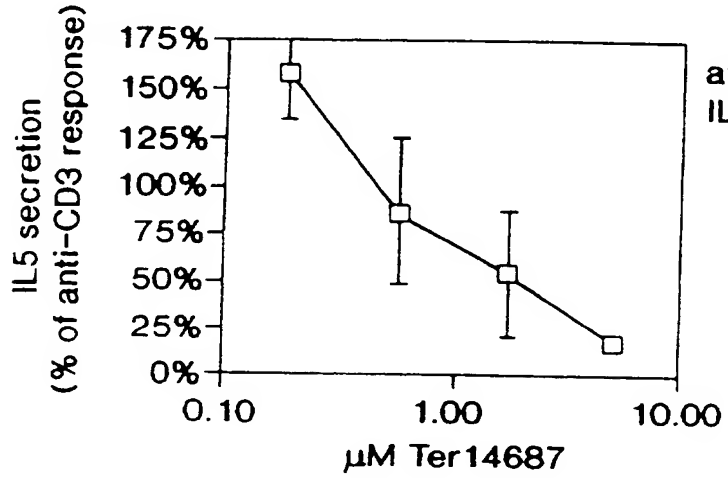


FIG. 13



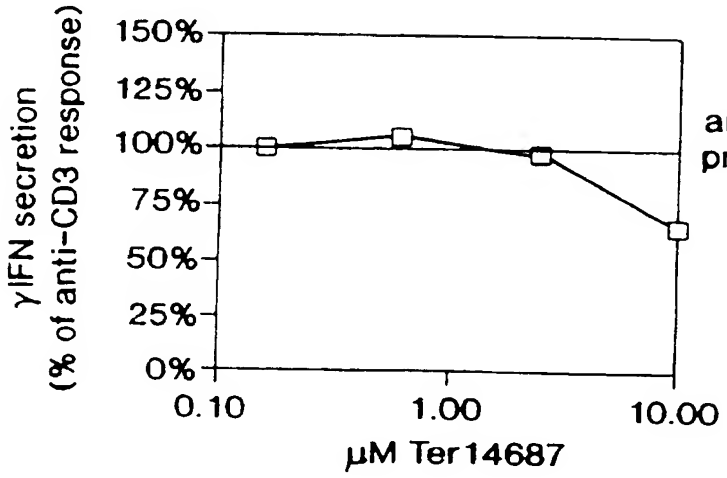
average anti-CD3 induced IL4 production = 60pg/ml

FIG. 14A



average anti-CD3 induced IL5 production = 400pg/ml

FIG. 14B



anti-CD3 induced γIFN production = 12411 pg/ml

FIG. 14C

2-10.SEQ

GCCTCAGCACAGCCCTTCCTCTGCTGCCTGTGCGGTATGATCTTTCCTGGGAGG
ACTGGCTACAGGCGTCATCTGCGCCAGGCTCATGGAGCTTCTGCCATGACTGA
GGGCTCAGAAGAAGAGGAGGAAGGCACAGCAGAAACAGCCTCTAC
CCATAGTCCTCCCCTGCAACTCTCAGAAGCAGAGCTGCTGAATCAACTGCAGC
GTGAGGTGGAAGCTCTAGATGGAGCAGGTTATGGTCATATTTGTGGTTGCTGT
GGTCAGACCTATGATGACCTGGGGAGCCTGGAGCGTCACCACCAAAGTCAAAG
TTCTAGCAATAGGACAGAGAATGTTCCTAGCCATTTGGAAGGAGCAGGTGATG
CAACAGAAATGGTTGCAGATCATGGCTTTGAGGGCACAGTGACCTCCGTCTCA
GAAGAAGGTGGGGACATAAAGTCTGAAGAGGGAGTAGGTGGCACAGTTGCAG
ACAGCCTTTGCATGCAGGCTGGTGAAAGCTTTCTGGAGTCCCACCCTCGCCCTT
TCCAATGTAACCAGTGTGGCAAGACCTATCGCCACGGAGGCAGCTTGGTAAAC
CACCGAAAGATCCACCAGACAGGTGATTTCATCTGTCCTGTCTGTTCCCGCTGC
TACCCCAATCTGGCTGCCTACCGGAATCATCTGCGGAATCACCCTCGCTGCAAA
GGCTCAGAGCCCCAAATGGGGCCCATCTCAGAAGCAGGAGGCTGCAGTGAGC
CCCAGAATGCAGCAGAGGCAGGGCAGGAGCAGGCTGTCATAGGGCAACTCCA
GGAAGAGCTTAAAGTGGAGCCCTTGGAGGAGCTGGCAGGTGTCAAAGAAGAA
GTGTGGGAGGGGACCGCTGTAAAGGAAGAGGAGCTGGAGCAGGAGTTGGAG
ACAGGCTGTCAGACTGAGGTCACCTCGGAGCGGCCCTTTAGCTGTGAAGTGTG
TGGCCGCACCTACAAGCATGCTGGCAGCCTTATCAATCACCGGCAGAGCCACC
AGACTGGCCA

2-10.PEP

ASAQPFLCCLCGMIFPGRTGYRRHLRQAHGASAMTEGSEEEEEGTAETASTHS
PPLQLSEAELLNQLQREVEALDGAGYGHICGCCGQTYDDLGSLERHHQSQSSSNRT
ENVPSHLEGAGDATEMVADHGFEGTVTSVSEEGGDIKSEEGVGGTVADSLCMQA
GESFLESHPRPFQCNQCGKTYRHGGSLVNHRKIHQTGDFICPVCSRCYPNLAAYR
NHLRNHPRCKGSEPQMGPISEAGGCSEPQNAAEAGQEQAVIGQLQEELKVEPLEEL
AGVKEEVWEGTAVKEEELEQELETGCQTEVTSERPFSCEVCGRT
YKHAGSLINHRQSHQT

2-32.SEQ

GGAAGCCAAGGGCACATCTCTATACCCCAGCCTGACTGCCCAGAGGAGGTGC
GGGCCTTCTCCTTCTACCTCTCCAATATTGGCCGCGACAGCCCTCAGGGCAGCT
TTGATTGCATCCAACAATATGTATCCAGCTATGGGGATGTACACCTGGACTGCC
TGGGCAGCATCCAGGACAAGGTCACGGTGTGTGCTACTGATGACTCCTACCAG
AAAGCACGACAGAGCATGGCACAGGCAGAGGAGGAGACTCGGAGCCGAAGT
GCCATCGTCATTAAGGCTGGAGGCCGATACATGGGGAAAAAGGTTCAGTTTCG
GAAGCCAGCGCCAGGGGCAGCTGATGCAGTACCCTCCCGGAAGCGTGCTACC
CCCATTAACCTGGCAAGTGCCATCAGAAAGAGCAGTGGGAGTGGAGCCAGCA
GTGTGGTACAGAGGCCCTTCCGAGATCGGGTGCTACACCTCCTGGCCCTGAGG
CCCTACAGGAAGGCTGAGCTGCTGCTGCGGTTGCAGAAGGATGGGTTGACAC
AGGCAGACAAGGACACCCTGGACAGCCTGCTGCAGCAGGTGGCCAGTGTGAA
CCCCAAGGATGGCACGTGCACGCTGCAGGACTGCATGTACAAAAGCCTGCAGA
AGGACTGGCCCGGCTACTCTGAGGGGGACCGGCAGCTGCTGAAGCGCATGCT
CATGCGGAAGCTGTGTCAGCCACAGAATGCCACTACAGACTCCAGCCCGCCCC
GAGAGCATGGACGCTCTGCCTCACCCTCTCAGAAACGGCGTACAGACTTCATT
GACCCCCTGGCCAGCAAGAAGCCCCGGATCTCACATTTCACACAGCGAGCACA
ACCCACCCTCAATGGCAAACTGGGTGCCCCCAATGGCCATGAGACACTGCTGC

FIG. 15A

CTGTTCCAGGACCCACCCCATCAGACACCTTCAGCTCTAGCCATCTGCCCCCAC
GGCTGGAGCCCCCAAGGACCCACGACCCCCTAGCTGATGTCAGTAATGACCTA
GGTCACAGTACCCAGGACTACAAGCACCAGGAAGCCACCCCAGCTCCAGCACC
CCATTTTGGTCTTCCCCTGCTGACGGACTTTCCTCAGGGTGAGCAACCTATTAG
TTCCTCACACACCCACAGCCGACCCAAGAAGAAGTCCAAGAAGCACAAAGACA
AGGAGCGGCCCCCTGAAGAAAGGCCCCCCGCCCCACAGCCTGATGCACCTACT
GCCCCTGCACTACCGCCAGATGCCCCAGGTCTGAATGGAGCCTGTGACAATGA
ACCCACATCCTTGTCAGAGACCCCGG

2-32.PEP

GSQGHISIPQPDCPEEVRAFSFYLSNIGRDSPQGSFDCIQQYVSSYGDVHLDCLGSIQ
DKVTVCATDDSYQKARQSMAQAEEETRSRSAIVIKAGGRYMGKKVQFRKPAPGA
ADAVPSRKRATPINLASAIRKSSGSGASSVVQRPFRDRVLHLLALRPYRKAELLLRL
QKDGLTQADKDTLDSLLQQVASVNPKDGTCTLQDCMYKSLQKDWPGYSEGDRQL
LKRMLMRKLCQPQNATTDSSPPREHGRSASPSQKRRTDFIDPLASKKPRISHFTQR
AQPTLNGKLGAPNGHETLLPVPGPTPSDTFSSSHLPPRLEPPRTHDPLADVSNDLG
HSTQDYKHQEATPAPAPHFGLPLLTDFPQGEQPISSSHTHSRPKKKSKKHKDKER
PPEERPPAPQPDAPTAPALPPDAPGLNGACDNEPTSLSETP

#10.SEQ

CGAGAACACACAGGCAAACCCACCACGAGTAGCTCAGAAGCATGTCGCTTCTG
TGGTTCCAGGAGTGGAACAGAGTTATCTGCTGTTGGCAGTGTTTGTTCTGATGC
AGATTGCCAGGAATACGCTAAGATAGCCTGTAGTAAGACGCATCCTTGTGGCC
ATCCATGCGGGGGTGTTAAAAACGAAGAGCACTGTCTGCCCTGTCTACACGGC
TGTGACAAAAGTGCCACAAGCCTGAAGCAAGACGCCGATGACATGTGCATGAT
ATGTTTCACCGAAGCGCTCTCGGCAGCACCAGCCATTCAGCTGGATTGTAGTCA
CATATTCCACTTACAGTGCTGTCGGCGAGTATTAGAAAATCGATGGCTTGGCCC
AAGGATAACATTTGGATTTATATCTTGTCCCATTTGCAAGAACAAAATTAATCAC
ATAGTACTAAAAGACCTACTTGATCCAATAAAAGAACTCTATGAGGATGTCAGA
AGAAAAGCCTTAATGAGATTGGAATATGAAGGTCTGCATAAGAGTGAAGCTAT
CACAACTCCTGGTGTGAGGTTTTATAATGACCCAGCTGGTTATGCAATGAATAG
ATATGCATATTATGTGTGCTACAAATGCAGAAAGGCATATTTTGGTGGTGAAGC
TCGCTGCGATGCTGAGGCTGGACGGGGAGATGATTATGATCCCAGAGAGCTCA
TTTGTGGTACCGAGAGCGTTTAGGTGAAACATATCATGCACATGTCATCGGCGT
CTTGCT

#10.PEP

REHTGKPTTSSSEACRFCGSRSGTELSAVGSVCSDADCQEYAKIACSKTHPCGHPC
GGVKNEEHCLPCLHGCDKSATSLKQDADDMCMICFTEALSAAPAIQLDCSHIFHLQ
CCRRVLENRWLGPRITFGFISCPICKNKINHIVLKDLLDPIKELYEDVRRKALMRLE
YEGLHKSEAITTPGVRFYNDPAGYAMNRYAYYVCYKCRKAYFGGEARCDAEAGR
GDDYDPRELICGTESV.VKHIMHMSSASC

1-22.SEQ

ATGGCGGCTGGGACCCTGTACACGTATCCTGAAAACTGGAGGGCCTTCAAGGC
TCTCATCGCTGCTCAGTACAGCGGGGCTCAGGTCCGCGTGCTCTCCGCACCAC

FIG. 15B

CCCACTTCCATTTTGGCCAAACCAACCGCACCCCTGAATTTCTCCGCAAATTTCC
TGCCGGCAAGGTCCCAGCATTTGAGGGTGATGATGGATTCTGTGTGTTTGAGA
GCAACGCCATTGCCTACTATGTGAGCAATGAGGAGCTGCGGGGAAGTACTCCA
GAGGCAGCAGCCCAGGTGGTGCAGTGGGTGAGCTTTGCTGATTCCGATATAGT
GCCCCCAGCCAGTACCTGGGTGTTCCCCACCTTGGGCATCATGCACCACAACA
AACAGGCCACTGAGAATGCAAAGGAGGAAGTGAGGCGAATTCTGGGGCTGCT
GGATGCTTACTTGAAGACGAGGACTTTTCTGGTGGGCGAACGAGTGACATTGG
CTGACATCACAGTTGTCTGCACCCTGTTGTGGCTCTATAAGCAGGTTCTAGAGC
CTTCTTTCCGCCAGGCCTTTCCCAATACCAACCGCTGGTTCCTCACCTGCATTAA
CCAGCCCCAGTT

1-22.PEP

MAAGTLYTYPENWRAFKALIAAQYSGAQVRVLSAPPHFHFGQTNRTPEFLRKFPAGKVPAF
EGDDGFCVFESNAIAYYVSNEELRGSTPEAAAQVVQWVSFADSDIVPPASTWVFPTLGIMH
HNKQATENAKEEVRRILGLLDAYLKTRTFLVGERVTLADITVVCTLLWLYKQVLEPSFRQAF
PNTNRWFLTCINQPQ

2-18.SEQ

AAAGCTTTAGAGGAGACCAAAGCCTATACAACCCAATCTCTAGCTAGTGTTGCT
TATCAAATAAATGCATTGGCCAACAATGTACTCCAGTTGCTGGATATCCAAGCC
TCTCAGCTTCGGAGAATGGAGTCTTCCATCAATCATATCTCACAGACTGTGG
ATATTCATAAGGAGAAAGTGGCACGAAGAGAGATTGGTATTTTGACAACAAATA
AGAATACATCAAGAACTCACCAAATAATAGCACCTGCGAATATGGAGCGCCCT
GTAAGGTATATTCGGAAACCTATCGATTACACAGTTCTGGATGATGTGGGCCA
TGGTGTCAAGCATGGAAATAACCAGCCTGCAAGAACTGGCACACTGTCGAGAA
CAAATCYTCCTAYTCAGAAACCGCCAAGTCCTCCCATGTCAGGCCGGGGAACA
CTGGGACGGAATACTCCTTATAAAACCCTGGAACCTGTTAAACCCCCACAGTTC
CTAATGACTATATGACCAGTCCTGCTAGGCTTGGAAGTCAGCATAGTCCAGGCA
GGACAGCATCTTTAAATCAGAGACCAAGGACACACAGTGGAAGTAGTGGAGGA
AGTGGAAGTCGAGAAAACAGTGGTAGCAGTAGTATTGGCATTCCCATTGCTGT
GCCTACACTTTCGCCACCCACTATTGGACCAGCAGCCCCGGGCTCAGCTCCTG
GTTTCCCAGTATGGCACAATGACCAGGCAGAC

2-18.PEP

KALEETKAYTTQSLASVAYQINALANNVLQLLDIQASQLRRMESSINHISQTVDIHK
EKVARREIGILTTNKNTSRTHQIIAPANMERPVRYIRKPIDYTVLDDVGHGVKHGN
NQPARTGTLSRTNXPXQKPPSPPMSGRGTLGRNTPYKTLEPVKPPQFLMTI.PVLL
GLEVSIVQAGQHL.IRDQGHTVEVVEEVEVEKTVVAVVLAFPLLCLHFRHPLLDQQP
RAQLLVSQYGTMTRQ

FIG. 15C

3-1.SEQ

CCGCCGCCTTTATTAGCTGAGCCATTACTTGAGAGGGATGAAGCGGGAGGAG
TGGGTGGCCCCGATGCCGGGCCGGCCATGCTTTACGGGCTTGTAGGTGATGG
AGAACTCGCCCAGGTAGTGGCCGATCATCTCGGGCTTGATCTCCACCTGGTTG
AAGGTCTTGCCGTTGTAGACGCCCACCATGCTGCCCACCATCTCGGGTAGGAT
GATCATGTCCCGCAGGTGCGTCTTCACCACTTCCGGCTTCTCCATGGGCGGCG
CCTCCTTCTTGGCCTTGCGCAGGCGCTTCAGCAGGGAGTGCTGCTTCCGCCGC
AGGCCCCGGTTCAGCCGCCGCCGCTGGCGCGCACTGTACAGCTGCATCAGCTG
CTCGTAGGACATGTCCAGCAGCTGGTCGAGATCCACGCCGCGGTAGGTGAAC
TTGCGGAAGGTCCGCTTCTTCTTCTGCTCTACTTCTGCCATCTTGCCGGCGGCC
GC

3-1.PEP

PPPLLAEPLLERDEAGGVGGPDAGPAMLYGLVGDGELAQVVADHLGLDLHLVE
GLAVVDAHHAAHHLG.DDHVPQVRLHHFRLLHGRRLLLGLAQALQQGVLLPPQA
PVQPPPLARTVQLHQLLVGHVQQLVEIHAAVGELAEGPLLLLLYFCHLAGGR

2-20.SEQ

GGGGATGCAGGCGTGGTCCTCCTCCAGGTCCTTCAGGCAGATCTCCAGGTGCA
GCTCGCCGGCGCCCGCGATGATGTGCTCTCCCGACTCCTCGATGATGCACTGC
ACCATGGGGTCGGACTTGGCCAGCCGCTTCAGCCCCTCCACCAGCTTGGGCAG
GTCAGCCGGGTTCTTGGCCTCCACGGCCACTCTGACAACAGGGCTGACGCTGA
ACTTCATCACCCGCATGTTGTGCGCGTGCTCGAAAGTGGTGATGGTGCCCGTC
TTCACCAGGAACTGGTCCACGCCCACGAGCCCACAATGTTCCCACAAGGCACA
TCCTCGATGGGCTCCACGTAtCGGGCCATCATCAAGATTGTTCTCTGGATTGGC
TTCAGGTAGAAGTCCTCCTCTTCCACGGGTTTTATTGGG

2-20.PEP

GDAGVVLLQVLQADLQVQLAGARDDVLSRLLDDALHHGVGLGQPLQPLHQLGQ
VSRVLGLHGHSDNRADAELHHPHVVRVLESGDGARLHQELVHAHEPTMFPQGT
SSMGSTYRAIIKIVLWIGFR.KSSSSTGFIG

FIG. 15D

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/16195

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/50 G01N33/68 C12N15/62 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,95 21252 (UNIV LELAND STANFORD JUNIOR) 10 August 1995 cited in the application see the whole document	1-4,6,7, 11,12
Y		5
A		18-22

Y	US,A,5 283 173 (FIELDS STANLEY ET AL) 1 February 1994 cited in the application see abstract	5

A	US,A,5 352 660 (PAWSON ANTHONY J) 4 October 1994 cited in the application see abstract	1,8,14

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
15 January 1997	31. 01. 97
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Authorized officer Ceder, O

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INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE WPI Section Ch, Week 9435 Derwent Publications Ltd., London, GB; Class B04, AN 94-026226 XP002022612 & JP,T,06 502 203 (NIPPON SHINYAKU CO LTD) , 4 August 1994 see abstract -----	23

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INTERNATIONAL SEARCH REPORT

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Int. onal Application No
PCT/US 96/16195

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